UNIVERSITY OF TORONTO
MEDICAL JOURNAL

NEW LINES OF RESEARCH AT THE ONTARIO CANCER INSTITUTE

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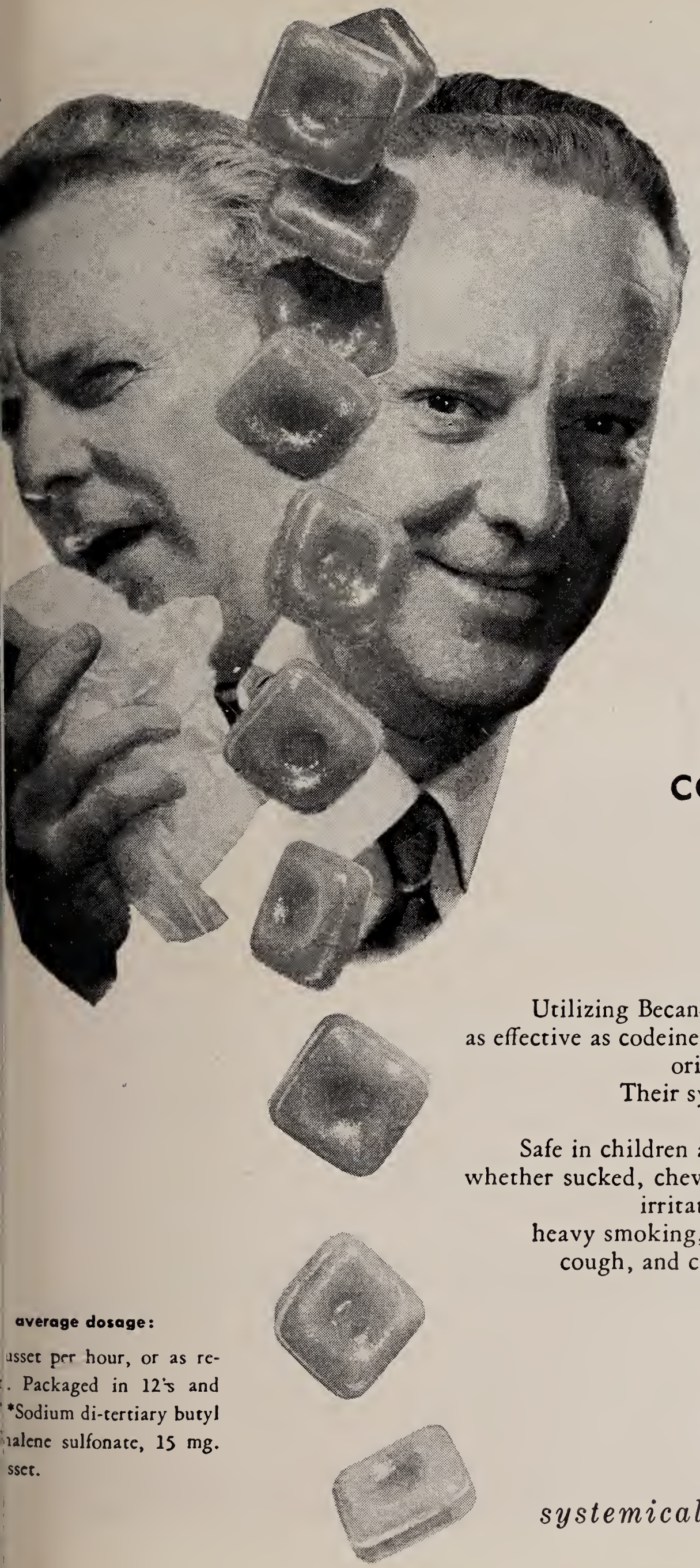

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EDITORIAL

In publishing the February issue of the Medical Journal we find ourselves in the enviable position of having a series of up-to-the minute articles written by some of the foremost authorities in cancer research and related fields. Considering the great interest the cancer problem has aroused in both medical people and the laity, the material contained in the following pages should prove highly stimulating—and indeed exciting—dealing as it does not only with cancer research but with other fields of medicine.

All of the articles in this issue were written by members of the staff of the Division of Biological Research of the Ontario Cancer Institute, especially for the Medical Journal. Most of these men are also members of the new Department of Medical Biophysics of the University of Toronto, of which Dr. Arthur W. Ham is the Professor and Head. This department is housed in the laboratories of the Divisions of Biological Research and Physics of the Ontario Cancer Institute, 500 Sherbourne Street, Toronto 5, Ontario.

Dr. Ham is well known to students at the University of Toronto who are fortunate to have lectures from him, and is known universally for his textbook, *Histology*. When approached for information on some of the latest advances in cancer research, he gave us his whole-hearted and enthusiastic support, and with his staff, prepared the material presented in this issue. We owe him, the members of his staff, and the Ontario Cancer Institute, our especial thanks.

Extra copies of this issue may be obtained for $1.00 by writing to the University of Toronto Medical Journal, Room 114, Medical Building, University of Toronto, Toronto 5, Ontario. The price of this issue is increased due to its larger size and the cost of printing the photomicrographs.

SOME RECENT ADVANCES RELATING TO VIRUS AND THE ETIOLOGY OF CANCER

ARTHUR W. HAM

CANCER, using the term in its broadest sense, is the disease that begins when one or more body cells become altered in some way so that their growth is no longer restrained by those factors that normally act to maintain a status quo in the cell population in the various parts of the body. The cells that become altered tend to lose some of the special morphological features which formerly distinguished them and come to resemble more the embryonic cells, which gave rise to that part of the body; for this reason cancer cells are said to be anaplastic, which means they have turned backward in their form. Cancer cells, moreover, are said to be autonomous also, which means they are independent and self-governing; this means that the reason for their continued growth lies in themselves. In other words, the growth of cancer cells, unlike the growth of normal cells in compensatory hyperplasias, or in repair phenomena, is not due to alterations in their enviroment, but to some hereditable change in themselves; this can be shown easily by transplanting them from one animal to another of the same pure strain, for they continue to grow in their new and otherwise normal host until they destroy it.

What is the cause of this strange behaviour of body cells? But first, what do we mean by cause? It is customary, in discussing causes to describe predisposing and exciting causes. Persisting diseases, moreover, have continuing causes. Probably in most long continuing diseases the exciting cause remains as the continuing cause, as, for example, in tuberculosis. But this is not always true, for there are some diseases that can persist and progress even though the exciting cause has disappeared. For example, streptococci in the body may excite glomerulonephritis but the latter condition may persist and progress long after the exciting cause has disappeared. All this is mentioned because in considering the cause of cancer it is helpful to distinguish between exciting and continuing causes.

In discussing the cause of cancer it is not unusual for authorities to state that there are many kinds of cancer and that, therefore, cancer must have many different causes. It is true that cancer can develop in many different parts of the body that are exposed to different environmental factors and that it can develop from a great variety of different types of body cells. Nevertheless, the pathologist who examines sections of cancer from these different parts of the body can scarcely escape the conclusion that no matter where cancer cells arise they all manifest the same general type of defect, one that is responsible for their manifesting increasing progression in the spheres of anaplasia and autonomy. It, therefore, would be extraordinary if there were a variety of continuing causes of cancer; surely the defect in all cancer cells is of the same kind and indeed this is generally believed to be the result of a somatic mutation. Hence, those who say that cancer has many causes must be thinking of exciting causes—of different factors such as chemical carcinogens, hormone imbalances, radiation, chronic irritations, and so on, which could possibly be involved to different extents in exciting cancer in different sites.

Where does virus fit into the etiology of cancer? It is now proven beyond any doubt that virus can cause some kinds

of cancer in experimental animals; indeed the most direct carcinogenic agent that has ever been observed is the Rous virus, for an injection of this into a suitable chicken immediately excites the formation of a neoplasm. That virus must be one possible exciting cause of cancer has been accepted for years and the general view has been that such tumor viruses as had been discovered probably acted in the same way as other carcinogenic agents were believed to act, namely, by causing somatic mutations. Recent work in the virus field, however, has raised the question as to whether or not virus may be implicated in cancer to a much greater extent than has hitherto been believed, and in considering some of this work, which will now be described, two questions should be kept in mind:

(1) Could virus be the exciting cause of all kinds of cancer, with the continuing cause being a somatic mutation; and

(2) Could virus be both the exciting and continuing cause of all kinds of cancer?

Some of the facts that have been elicited and which may bear on these intriguing questions will now be given in the story of development of knowledge about virus and experimental cancer.

The Development of Knowledge about the Relation of Virus to Tumors of Experimental Animals

In trying to establish whether any given disease is due to a virus the first milestone was generally considered to have been passed when an example of the disease could be produced in a susceptible animal with a cell-free filtrate. Accordingly, when Rous, in 1911, showed that sarcomata were almost immediately induced at sites where chickens were injected with cell-free filtrates prepared from other chicken sarcomas, it might have seemed that the first milestone had been passed and that it would be only a matter of time before other, and finally all varieties of malignancy would be shown to be caused by virus.

But, for many years, attempts to induce other types of neoplasms with cell-free filtrates of neoplasms were, in general, most discouraging. True, there were occasional successes to keep the hopes of the virus proponents alive. The discovery of the milk factor or agent and its role in causing mammary tumors in mice was particularly stimulating because it illustrated that a tumor virus, taken orally by a new-born mouse, could seemingly remain latent for a long time and then act later in life (but only in certain strains of mice and in conjunction with the action of hormones that stimulate mammary gland growth and function) to induce neoplasia. The discovery of the Shope papilloma virus, the virus of the Lucke carcinoma of the kidney of the frog, and the establishment that various forms of the leukosis complex of chickens were due to virus, and that the latter could be transmitted through the egg, were all encouraging to the proponents of the virus school. But it must be admitted that for four decades after Rous made his discovery there was still no substantial indication that malignant neoplasms in general could be induced with cell-free filtrates or that they were in any way associated with a virus.

In the meantime a great deal of information was accumulating about the induction of cancer with chemical carcinogens, of which a great many were found, and radiation, and since these agents are generally mutagenic as well as carcinogenic, the experimental work on carcinogens tended to re-inforce the old hypothesis that cancer represents a somatic mutation. And, although it was admitted that there were a few tumors that were caused by virus, these were dismissed by most authorities as curiosities and the great bulk of opinion was that cancer in general was not a virus disease.

Recently, however, several findings in several fields have converged to provide the basis for a re-examination of the virus hypothesis. These new findings, in different fields, are not easily presented in chronological order, so they will be introduced as they relate to the discussion at hand.

Gross, in 1951, showed that cell-free extracts prepared from the leukemic tissue of mice of a strain characterized by a high incidence of spontaneous leukemia (AK) would, if they were injected into *new-born* mice of a low leukemic strain (C3H), cause leukemia to develop in the injected mice some months afterward. Extracts made from the leukemic tissues of these would induce leukemia if they were similarly injected into new-born mice of the same low leukemic strain. These results have been abundantly confirmed.

Shortly afterward both Gross, and Sarah Stewart, who was also working with the same type of extract, made the further and most interesting observation that some of the new-born mice injected with the extract of leukemic tissues developed parotid gland carcinomas and/or fibrosarcomas of their connective tissue.

Next, Sarah Stewart introduced cell-free extracts prepared from leukemic mice into tissue cultures, first of monkey kidney cells, but later, cultures of mouse embryo cells. The virus reproduced in both these cultures and produced a cytopathogenic effect on the mouse embryo cells. Cell-free extracts prepared from the infected cultures, injected into new-born mice, resulted in the injected mice subsequently developing many kinds of solid tumors. Furthermore, the agent or agents in the extracts showed the ability to cross species barriers, and produced various tumors when new-born animals of certain other species were inoculated with them. The fact that the agent or agents in the extract that originally induced leukemia plus occasional parotid tumors or occasional sarcoma when injected directly into new-born mice would, after its cultivation in tissue culture, produce a broad spectrum of tumors, is a very impressive finding. Anyone who has seen an animal, injected with virus at birth, literally explode into a whole group of tumors some weeks or months later, can scarcely escape the development of interest in the virus hypothesis.

Although we will later discuss experiments of both Gross and of Stewart with older animals, it is obvious that the use of the new-born animal was of key importance in enabling them to make the discoveries described above. This fact makes us recall that the Bittner milk agent, to be properly effective in producing mammary tumors of mice must be administered to very young animals. In nature it is received by new-born mice at their first feeding.

What is probably the reason for the successes obtained by injecting new-born animals with tumor agents is suggested from investigations made along lines other than cancer. One step in these investigations was the discovery that the tissue of unlike twins, who had shared a common circulation during embryonic and fetal life, and so were exposed to each other's antigens before birth could, in postnatal life, be successfully grafted from one to the other. Continued work in this field has revealed that antigens given to feti do not invoke an antibody response but instead seem to be accepted as normal components of the developing animal, with the result that after birth, the animal can never effectively make antibodies against these antigens when it is challenged with them; such animals are said to manifest "induced tolerance" to these antigens. The period during which an animal can be made tolerant to an antigen ends usually around the time of birth but varies somewhat in different species. The period of tolerance

to antigens is followed by a brief period where antigens neither induce tolerance nor invoke antibodies and this, in turn, is followed by the final period when antigens incite antibody formation; this final period usually begins shortly after birth but may begin before birth in some species.

Now it might seem at first thought that the importance of the work of Gross and of Stewart lies in the fact that they have proven that some further animal leukemias and cancer can be transmitted by a virus or viruses which sometimes cause leukemia and sometimes solid tumors of different types. But, on second thought, it might be concluded that they have shown something that is important in relation to the development of leukemia or cancer under ordinary conditions; namely, that it is the inability of an animal's immunity mechanism to effectively resist a tumor agent that is the important factor in the development of the condition. An impressive illustration of this is provided by Gross who has shown that extracts of the tissues of normal C3H mice, of a variety free from milk factor, when injected into new-born mice of the same strain, results in the latter developing later a significant proportion of parotid tumors. Apparently the important factor here in determining whether or not the donor or recipient mice developed parotid tumors, was *not* the presence of agent for *both* had that but, instead, whether or not the mice had the ability to resist the agent; this was not possessed by the mice injected as new-borns but was possessed by the normal animal from which the virus was obtained.

If we can reason from mice, this leads to the very interesting conclusion that, under normal conditions, the important factor in whether or not leukemia or tumors develop is not the introduction of an agent into the organism, but the state of the immunity mechanism with regard to resisting the agent or agents. The experiments of Wooley seem to suggest the same reasoning for he showed that otherwise normal mice given cortisone over long periods which, of course, would seriously depress their immunity mechanism, resulted in a significant proportion of the mice developing parotid tumors. These experiments again would seem to suggest that it is a defective immunity mechanism, and not the presence of virus that is the decisive factor under ordinary conditions. The experiments of Kaplan might be mentioned here also. Kaplan showed that C57 mice, a variety which ordinarily do not show a high incidence of leukemia, develop the condition after certain fractionated doses of radiation. The leukemia generally begins in their thymus glands. He has shown, moreover, that if he transplants a thymus from a non-radiated isologous mouse into a thymectomized radiated mouse, leukemia still occurs, and may begin in the cells of the non-radiated transplanted thymus. Furthermore, if he injects bone marrow from an isologous mouse into a radiated mouse after its last dose, leukemia does not generally develop. Since in the experiments with transplanted glands the leukemia develops in non-radiated cells the condition could scarcely be explained as a somatic mutation resulting from the mutagenic action of X-rays. Instead, since the amount of radiation given would greatly impair the immunity mechanism of the host, the development of leukemia in these radiated mice could be explained by the inability of the radiated animal to resist some tumor agent present within it. Likewise the protection of the radiated animal against leukemia by the intravenous injection of isologous bone marrow could be explained by the new marrow providing a basis for a new unimpaired immunity mechanism in the host.

The above-described experiments, and others to be described presently, indicate

that tumor agents are not uncommonly at least temporary residents of at least some animal organisms under ordinary conditions, and that they can be present without causing leukemia or tumors providing the immunity mechanism of the host is effective enough to prevent them from inciting disease. Accordingly, in trying to determine the significance of experiments in which virus is injected into new-born animals, with them later developing the disease, it must be appreciated that these experiments show something much more than the fact that the injection of a virus will cause disease (which could be interpreted as an experimental procedure not related to the development of the disease in man). The experiments show something further that may be important in relation to man; namely, that an inability of a host to resist tumor virus (this can be accomplished experimentally, for example, by injecting a new-born animal or by total body radiation) is a very important factor in permitting disease to develop—and this could be true of man.

If tumor virus appears spontaneously in the animal organism where does it come from? The phenomenon of lysogeny, discovered from studies of bacterial viruses, may perhaps provide the clue. Microbiological studies have shown that certain bacterial viruses may exist as proviruses in the chromosomal structure of bacteria, where they exert some definite gene effect. But either spontaneously, or under the influence of certain activating agents, they can become detached from the chromosomal structure and embark on careers of being free destructive viruses, bringing about, for example, lysis of the bacteria of which they were a part, and lysis of other susceptible cells that they subsequently infect. But, under certain circumstances, on infecting bacteria they may again enter into the chromosomal structure of the organisms, and when they do so they cease acting as free agents, to become actual parts of the chromosome and to which they contribute certain genes. It is also of interest here to mention that it has been shown that several chemical carcinogens as well as radiation are good activating agents for lysogenic systems, and this suggests the possibility that all carcinogenic agents may act through the common path of liberating free tumor virus. It is of interest in this respect that of all the carcinogenic agents studied so far, the Rous virus has the most direct effect, for as has already been mentioned, an injection of Rous virus into a chicken immediately incites the malignant process. Chemical and physical carcinogens act only after a long latent period which suggests they act indirectly.

The phenomenon of lysogeny has only been observed so far in connection with DNA viruses and in bacteria. As yet we do not know whether tumor viruses are of the DNA or the RNA type. The only one about which definite information has as yet been obtained is the Rous virus and it is of the RNA type. Nevertheless, lysogeny is a biological precedent and even though the existence of this mechanism has as yet only been elucidated in connection with certain DNA viruses in bacteria, the possibility of similar phenomena in mammalian cells and related to both DNA and RNA viruses must be taken very seriously. In any event, the fact is that tumor virus has been recovered from normal mammals which did not have cancer and which would probably never have developed the disease. In addition, it is now known that tumor virus can be recovered from malignant disease in mammals incited by radiation and the significance of this very impressive finding will now be discussed. Gross has shown recently that C3H mice (without milk factor) and which ordinarily have a low incidence of leukemia, will develop leukemia in large numbers some months after they receive fractio-

nated doses of radiation. He has shown, moreover, that cell-free extracts, prepared from the leukemic tissues of such leukemic mice will, if they are injected into new-born mice of the same strain, induce leukemia in them. Members of our group, moreover, as is described in the article on electron microscopy, have found virus in a parotid tumor that developed in a C3H mouse given 800 r of radiation and subsequently protected by rat bone marrow.

The establishing of the virus etiology of spontaneous leukemia in mice, the discovery that cell-free extracts from leukemic tissue given to new-borns sometimes incite other kinds of tumors instead of leukemia, the finding that the inoculation of cells in tissue cultures with these cell-free extracts results in an agent or agents which cause a whole spectrum of tumors when injected into new-borns, and that this agent or agents will cross species barriers and produce this same effect, and the finding that tumor virus can be recovered from normal animals and from leukemic tissues of mice in which leukemia is incited by radiation, all suggest that the role of virus in the etiology of malignant disease may be more general than has hitherto been assumed. Indeed, these facts raise the questions mentioned earlier—so far as mouse tumors are concerned—as to whether virus is not the exciting or even the continuing cause of all mouse tumors.

If virus were merely the exciting cause of certain mouse tumors, and acted only to induce a mutation, there would be no need for virus to persist in tumor cells to ensure their continued malignant behaviour for this would be accounted for by the mutation induced by the virus. It has, therefore, always been puzzling—if virus is only an exciting cause—that virus can be recovered from tumors incited by viruses even though these tumors are transplanted through many generations. It could be reasoned, of course, that the virus was not necessary in these instances and was only being carried along as a passenger in the proliferating cells. But, nevertheless, this fact raised the possibility in some minds that virus might be the continuing cause of the malignant process in these instances. Now that virus has been recovered from tumors induced by radiation the case for virus being a possible continuing cause of cancer is much stronger.

It might be thought that the problem of whether virus is the continuing cause of all cancer could be settled easily with the electron microscope. It is true that virus has been found, by this method, in cells of tumors that began in radiated animals. It is true also that the cells of many kinds of tumors do not reveal virus by this method. This brings up a problem, however, of whether we as yet can recognize virus particles in cells in all stages of their life cycles. It seems probable that during certain stages of their life cycles in cells they do not exhibit the appearances that have, as yet, been accepted for their identification with the electron microscope. For example, the most important criterion by which virus is identified with the electron microscope is that it is a rounded body within a certain small size range which consists essentially of a membranous shell which is generally double layered. Within cells the membranous shell may or may not possess denser material in its interior but in extracellular virus a relatively dense nucleoid body can generally be seen.

The membranous shell of the virus particle bears a striking resemblance to a microvesicle of the golgi apparatus of the cell; and indeed, it is by no means certain that at least one of the shells of a virus particle is not derived from the golgi apparatus. It is assumed that the nucleoprotein component of virus is contained within the membranous shell; credence is given this view by the dense appearance of the nucleoid body in extra-

cellular virus and in the instance of Rous virus the nucleoid body can be removed by ribonuclease. It may be, however, that this more or less classic picture of virus, may be that of virus that has been segregated (perhaps in a golgi microvesicle) in the cytoplasm of the cell as a preliminary step to its extrusion, and that it may have phases of existence in cytoplasm when it is not segregated from cytoplasm, and hence has no membrane surrounding it. And, if it were an RNA virus, and had no surrounding membranous shell it would, of course, be very difficult, if not impossible, to distinguish the granules which might later become aggregated into a nucleoid body from cytoplasmic RNA granules of approximately the same size.

In general the RNA granules in the cytoplasm of tumor cells are more diffusely distributed than in normal cells and fewer of them are attached to intracytoplasmic membranous vesicles. If it should prove that the tumor process is primarily due to RNA granules that are altered qualitatively, and that such an alteration can occur spontaneously, by a process somewhat similar to lysogeny, or by the cell becoming infected with an extraneous RNA tumor virus, and that within the cell altered RNA granules are sometimes segregated into vesicles to give the classic appearance of virus, and sometimes not, we would have a situation where the boundaries between virus and mutation were not very clear cut. And this may be where the answer lies.

In the light of this new evidence what are the possibilities? The use of the newborn animal and electron microscopy have shown tumor virus to be present where its presence was not acknowledged before. The discovery of lysogeny provides a clue as to how tumor virus could appear within an organism. It is possible that chemical and physical carcinogens only act as predisposing causes to increase the rate at which provirus is activated, and that tumor virus is always the exciting cause of cancer. It may be that tumor virus affects the chromosomes so that a particular type of mutation makes its appearance. On the other hand, it is possible that the nuclear changes in cancer are not causative but secondary to the metabolic disturbance occasioned in the cell by an altered RNA. And, if altered RNA rather than altered DNA is the continuing cause of cancer some new criteria may have to be evolved to decide whether the altered RNA should be thought of as mutant RNA or virus. Fortunately, these various hypotheses lend themselves to investigation in the laboratory and are in a good part responsible for the fact that cancer research is now more stimulating that at any time in its history.

ACTIVELY ACQUIRED IMMUNOLOGICAL TOLERANCE

I. M. CASS

WHEN an antigenic substance is injected into an *adult* animal, an immunological response is elicited, i.e., antibody is formed, and this antibody may establish a state of protection against the same antigen or may induce hypersensitivity. However, when an antigen is injected into a *fetus*, no antibody is formed. Furthermore, as a result of being inoculated with an antigen during fetal life, an animal is unable to react normally to subsequent doses of the same antigen given in postnatal life; its capacity to make antibody to this antigen is

weakened or suppressed. The state of specific unresponsiveness to a particular antigen developed in this manner, is called "actively acquired tolerance".

It should be emphasized that this state is specific for the particular antigens to which a fetus is exposed, and in general the animal's reactions to other antigens are left unimpaired.

The period of time during which actively acquired tolerance may be induced generally ends near the time of birth. This alteration in the animal's ability to react, is one of the fundamental changes associated with the transition from the fetal to the postnatal state. In adult life the administration of foreign antigens results in the production of immunity rather than actively acquired tolerance. However, between the time when antigens produce tolerance, and the period when they produce immunity, there is a brief "null" or changeover period when an antigen produces neither tolerance nor immunity. The precise time at which an antigen no longer produces tolerance varies between species and depends on the nature of the antigen employed. For example, tolerance can be induced in the mouse to a homologous skin transplant only up to the first day of postnatal life, but in the rat, it can be induced, at least sometimes, up to the fifteenth day. In the calf, however, the period in which tolerance to a skin transplant can be induced, is over by the time of birth. But, whereas tolerance to a skin transplant can not be induced in a calf at the time of birth, tolerance to trichomonas fetus antigen can be induced up to the age of four weeks (3).

Owen in 1945 was the first to observe in nature the phenomenon of acquired immunological tolerance. Twin calves usually share a common circulation in foetal life because of anastomoses between their placental blood vessels. Owen found that in non-identical twin calves, after birth, each calf contained a mixture of serological types of erythrocytes, its own serological types and those of its twin which were foreign to it. Since these "foreign" red cells were found in the circulation far beyond the life span of an erythrocyte, it appeared that red cell precursors had traversed the placental connections, and established themselves in the opposite twin, where these hematopoietic cells continued to form erythrocytes that were serologically different from the red cells of their host. This was the first observed example of cells from one fetal animal establishing themselves in a second animal, and being accepted by their new host without antibodies being formed against them or their progeny, even after the host reached the adult state.

In 1951 Billingham, Brent and Medawar, found that the majority of non-identical twin calves would accept transplants of each other's skin regardless of differences of sex or colour, whereas, as was expected, transplants between siblings of independent birth were rapidly rejected. They named this phenomenon of acceptance of a transplant from a donor animal "tolerance" and Billingham and his associates have subsequently contributed much to the understanding of this phenomenon. They showed that the phenomenon which they observed to occur in twin calves by natural accident, could be reproduced in laboratory animals. By injecting fetuses of mice, rats and rabbits and embryos of birds, with foreign cells, they were able to produce a state of tolerance in the host to the cells of the donor. The induced state which permits donor tissue to be accepted by a host because of its having been injected in fetal life with cells of the donor, they termed "actively acquired tolerance." In investigating the fundamental principles of this phenomenon, they chose skin as the "test dose" tissue, i.e., the tissue which indicates whether or not the host is tolerant to the cells of

the donor, for the changes produced by increased or decreased tolerance are readily observed in transplanted skin by outward inspection. Occurrence in the transplant of partial baldness, contracture or eczematous deterioration, are sensitive criteria of diminishing tolerance, whereas a healthy appearance indicates full acceptance, i.e., well established tolerance. Mice were chosen for most of the experiments, because a large variety of inbred strains is available. The graft, whether skin or other tissue, may have one of three possible fates: (1) It may not last as long as a graft in an untreated animal, indicating that the first injection produced immunity. (2) It may survive beyond the known survival time in an untreated animal, indicating that the first injection induced tolerance. (3) The survival time may be the same as in an untreated animal, indicating that neither tolerance nor immunity resulted from the first injection.

The work of Billingham and his coworkers has been largely confined to tolerance induced by living tissue. Other workers have experimented with different types of antigens, and found them capable of producing actively acquired tolerance. Bacterial antigens are able to induce tolerance as shown by Buxton in 1954 (11). Killed organisms of Salmonella Pullorum, injected intravenously into chickens up to the age of 15 days, reduce their power to resist infection. Protozoa and their products, as in the previously cited example of trichomonas fetus antigen, can induce actively acquired tolerance (3). In guinea pigs picryl chloride is able to produce tolerance by skin contact (1). Hanan and Oyama (13) and Cinader and his coworkers (12, 14, 22) have produced tolerance to foreign protein. The adult animal fails to produce precipitating antibodies to the specific protein antigen to which it is tolerant.

Traub in 1939 (15) showed that mice exposed in utero to the virus of lymphocytic choriomeningitis, could not form neutralizing or complement fixing antibodies to this virus when they reached adult life. Burnet and Fenner consider this experiment to be a valid example of actively acquired tolerance to a viral antigen. The tolerant mouse harbours the virus, for it can transmit the infection to a non-tolerant animal, but its own cells do not react to the virus and hence show no manifestations of the disease. It would appear that symptoms of the disease are not caused by the virus itself, but rather by the reaction of the body cells to the virus. In a tolerant animal, in which the reaction of the cells is suppressed, the disease does not manifest itself, even though the virus is intact. Tolerance can also be produced to Rous chicken sarcoma. Turkeys injected in embryonic life with chicken blood become susceptible to the virus of the chicken tumour (Simonsen 1955) (16).

The proof that tolerance affects the antibody forming mechanism and does not depend on local factors, is found in the method of abolishing tolerance (1). Immunologically competent cells from an animal which is immune to a specific antigen are capable of abolishing tolerance in an animal which is tolerant to the same antigen. Both animals must be of the same strain in order that the cells from the immune (donor) animal establish themselves in the tolerant (host) animal, and continue to produce antibody for the purpose of reversing the host's tolerance. Billingham, Brent and Medawar used lymph node cells for this purpose. A slower, but equally effective method, of abolishing tolerance, is to inject lymph node cells from a non-immune non-tolerant (donor) animal into a tolerant (host) animal of the same strain. These injected cells will grow in the host, and since they were not present in the host at the time of induction of tolerance, will produce antibody to the

previously innocuous antigen, and reverse the host's state of tolerance.

The fact that the cells of the animal organism co-exist in harmony and are not antigenic to each other, is a universal illustration of acquired immunological tolerance. There are tissues which are able to immunize the animal which bears them. Spermatozoa do not occur in the fetus, and are developed after the time when tolerance can be induced. Hence, they are auto-antigenic. The lens of the eye is nourished by the aqueous humor and is not in direct contact with the vascular system, either in the fetal or adult animal. For this reason, lens material can act as an antigen to its own bearer. These two exceptions to a general rule, emphasize the importance of tolerance in the development of life beyond the stage of the single celled organism.

Acquired immunological tolerance is manifested by the inability of a tolerant animal to produce antibody. Fagraeus (17) and Coons, Leduc and Connolly (18) have shown that antibody is formed by plasma cells in the process of their maturation. Plasma cells do not occur in fetal life (19), the period when tolerance to an antigen can be induced. The possibility that the basis for the phenomenon of actively induced tolerance, lies in the inability of plasma cells to mature in response to stimulation by the specific antigen to which the animal is tolerant, may be the basis of the phenomenon of tolerance.

The practical application of actively acquired tolerance to the fields of allergy may be attempted in the near future; there are, however, some practical difficulties in the way. In Billingham and Brent's experiments (20) some mice rendered tolerant to skin homografts, manifested involution of lymph nodes, fibrosis of the spleen, progressive weight loss, coarseness of hair, and a short life span. They called this phenomenon "runting" and believed it to be due to a reaction between the graft and its tolerant host. Although "runting" may not occur with tolerance to defined purified antigens, its existence indicates the need to exercise caution in the possible applications to medicine of the principles of actively acquired immunological tolerance.

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THE NATURE OF VIRUS MULTIPLICATION

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THE role of viruses as causative agents of disease has been evident for many years. Less evident, but none the less important, is the role that viruses have played and are playing in obtaining information about many of the most important fundamental problems in biology. Viruses are simple structures. However, simple though they may be, viruses have attracted the attention of scientists of many different backgrounds, physicists, chemists and biologists, because they exhibit many of the important properties of all living matter, such as the ability to replicate and mutate. Because of these latter properties, viruses are able to show variation, inheritance, and selection in a manner similar to higher organisms. In addition to their own genetic variation, certain viruses can become modified in a cell so that they become part of the bacterial chromosome, and therefore achieve an intimate association with the genetic material of the cell. In so doing, these viruses can alter the genetic properties of the cell in various ways, as we shall see later.

No one has yet adequately defined a virus. For present purposes, it will perhaps suffice to say that viruses are submicroscopic units containing nucleic acids and proteins. Unlike bacteria which can multiply and grow freely in nature, viruses require the cooperation and metabolic machinery of a cell in order to propagate. This means that viruses grow only when they are inside a cell.

Viruses are classified in relation to the cells in which they multiply. For example, bacterial viruses are those that grow in bacterial cells, and animal, plant and insect viruses grow in animal, plant and insect cells respectively.

Multiplication of the virus within a

cell is usually pathogenic to that cell, and this provides a basis for determining the presence of a virus.

The destruction of cells by the virus particles has led to the development of a beautiful titration method, called the plaque (French for patch) assay method. This technique, used originally for the bacterial viruses, and later for the animal viruses, takes advantage of the ability of virus to multiply in and to kill and lyse host cells. Experimentally, the virus particles to be assayed are placed in contact with a very large number of cells in a Petri dish. The number of cells is such as to form a confluent or continuous cell sheet across the whole area of the Petri dish. Each virus particle in contact with the sheet of cells, will infect one cell. This cell will produce about 200 virus particles before dying, and these 200 virus particles will then infect 200 more cells in the near vicinity. This will then lead to the necrosis of 200 cells and so on. Diffusion of these liberated virus particles to cells far removed from the original focus of infection is prevented by carrying out the whole process in solid agar sheets. It can be seen that a steadily increasing area of necrosis will be observed as the virus makes its way, so that finally, holes, or plaques, in the sheet of cells will be observed and the presence of virus can thus be ascertained. Under suitable conditions, each area of necrosis started by a single virus particle can be distinguished as a plaque, and the number of plaques yields an estimate of the original concentration of virus.

The best understood group of viruses, perhaps because they are easiest to handle experimentally, are the bacterial viruses; these are called bacteriophages. The bacterial virus-bacterium system has been regarded by many in this field as a model system from which general conclusions about multiplication of viruses and macromolecules (such as proteins and nucleic acids) may be drawn. This article will therefore draw mainly on information obtained with the bacterial viruses to illustrate the main features of the biology of viruses.

Bacterial viruses are made up of only 2 components, protein and deoxyribonucleic acid (DNA). Each virus particle consists of a head which is often polyhedral or spherical in shape, and a tail (Fig. 1). A full cycle of viral multiplication involves many steps. First the cell is infected by the virus. The virus then disappears as an infective unit, and even if the interior of the cell is examined no virus particles can be found. This is called the eclipse phase. Soon new virus particles make their appearance inside the cell and when this number reaches about 200, the cell bursts open and releases these bacteriophages into the medium. Two hundred or more progeny virus particles are made from the original single infectious parental unit; the whole process takes about 20–30 minutes.

If this process is now examined in more detail, it is found that the virus first becomes attached to the bacterium by means of the tail. The next step that occurs is one of the most important facts in bacteriophage biology. *The virus itself does not enter the host bacterium,* but it acts as a syringe, with the tail as the needle, and only its nucleic acid, DNA, is injected into the cell. In effect, the nucleic acid in bacteriophage nucleoprotein is separated from the protein in this way. Little or no protein enters the cell, and since this infected cell will eventually produce many new virus particles, this means that protein is not involved in initiating the further steps of viral synthesis. This also makes it probable that it is the DNA in the bacteriophage particle which maintains genetic continuity from parent to progeny particles during multiplication, since injection of DNA alone into the cell suffices to produce 200 or so copies of the original infecting unit. Finally, the separation of

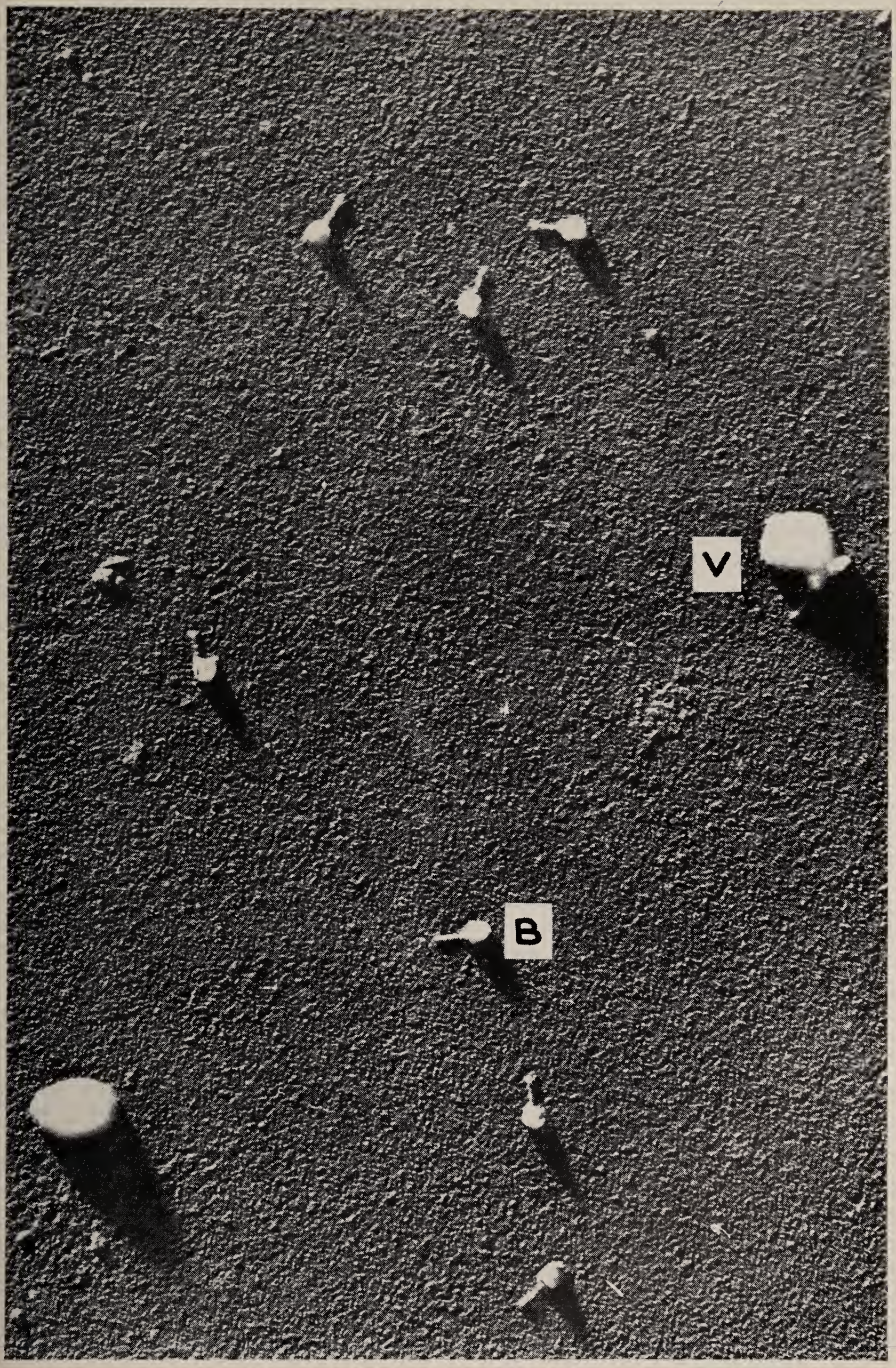

FIGURE 1. Electron micrograph of T2 bacteriophage (B) and vaccinia virus (V). X 35,000

nucleic acid and protein necessarily means that no mature virus particles will be seen in the cell immediately after infection, and this, of course, is what results in the eclipse phase mentioned earlier.

After a bacteriophage injects its DNA into a bacterium, the virus enters the eclipse phase and a number of different types of response can occur in the cell, of which the following two are the most important.

(1) Vegetative multiplication of bacteriophage building materials may occur leading to maturation of virus and lysis of the cell; this is called the productive response.

(2) The viral DNA may become integrated in the cell by becoming a part of the DNA of the bacterial chromosome. No vegetative multiplication occurs, and the cell survives. Such bacteria are called *lysogenic bacteria*, and after infection as described above, every such bacterium carries as part of its chromosomal content a genetic unit, called *prophage*. This prophage confers on these cells the ability to produce virus from within themselves without further exogenous infection; this process of lysogenization is called the reductive response.

Which of these two responses will be observed depends on environmental factors as well as the type of bacteriophage. *Virulent* bacteriophages can only give a productive response and *temperate* can give both.

Early in 1940, a number of investigators in various parts of the world decided to select a very limited group of bacterial viruses for more intensive examination. The group chosen were E. coli virulent bacteriophages with particularly favorable properties, and these viruses were called T1, T2, - - - - T7. Of these, T2 bacteriophage has been studied most intensively, and the following discussion of the productive response refers mainly to work carried on with T2.

Although no virus particles can be found in the cell during the eclipse phase of productive development, this is not a period of inactivity. Infection with the virus profoundly alters the metabolism of the cell and extensive biochemical and genetical events are occurring.

Immediately after infection with a virus such as T2 the cell stops dividing and the synthesis of respiratory as well as most other enzymes is completely inhibited. However some protein synthesis continues, and then, after a short interval, the synthesis of DNA commences. This new DNA can be shown to be DNA that will eventually be incorporated into the new virus particles. The early protein synthesis is not viral protein and will not be incorporated into the final bacteriophage particles. This period of protein synthesis is an important part of bacteriophage multiplication, however, because, if such synthesis is inhibited, no bacteriophage DNA will be made. The proteins that will be incorporated into the new mature virus particles only make their appearance late in the eclipse phase. The synthesis of bacteriophage DNA and protein seems to occur independently and their incorporation into a complete virus occurs only at maturation. No increase in ribonucleic acid (a nucleic acid not found in bacteriophages) is observed, although the RNA already in the cell remains metabolically active. Bacteriophages are not released from cells by secretion, but instead, after a large number have formed, the cell undergoes lysis with liberation of all the virus particles it contained.

Infection, injection, eclipse or vegetative phase, maturation and lysis, constitute one complete growth cycle in the productive response. The progeny bacteriophages that are produced at the end of this cycle are generally identical to the original parental infecting particle. However bacterial viruses are capable of genetic variation and progeny particles different from the parent can be produced in 2 ways: (1) by mutation, and,

(2) by recombination, as will now be discussed. Mutations occur in bacterial viruses only when they are multiplying in the cell and not while they are in the resting state outside the cell. Various types of mutations that affect different phage properties can occur, such as the nature of the plaques they will form, and the range of host cells which they will be able to infect.

Recombinations occur when a host cell is infected with a mixture of 2 related viruses of different genetic constitution. The mixture of progeny so obtained consists not only of particles identical with parental types but also of particles containing mixed properties derived from both parents. Genetic recombinations of this type have been observed and studied in many different types of bacteriophages. With some of the bacterial viruses, the study has gone far enough for a chromosome linkage map to be drawn similarly to the way such maps have been developed for higher organisms.

Genetic recombination occurs during the eclipse phase. The present evidence indicates strongly that the genetic material concerned in mutation and recombination in the eclipse phase is DNA. Because of the relative simplicity and accuracy of bacteriophage studies, a number of investigators have tried to draw clues from the process of bacteriophage multiplication involving genetic DNA units, to illustrate some basic principles concerning all replication processes.

Infection with a *virulent* virus always gives the productive response, leading to multiplication of the virus and death of the cell. A temperate virus can also give the productive response. However, occasionally, a temperate virus will behave differently leading to what has been called *lysogenization*, or the reductive response. When this occurs the virus DNA is modified after it enters the cell so as to become integrated into the bacterial chromosome. Such cells survive the infection, are able to multiply, and are called lysogenic cells.

If lysogenic cells are disrupted by artificial means, no virus can be recovered from them; the original infecting virus has disappeared as such. However, the cell retains a memory of the original infectious particle in the form of a gene on the bacterial chromosome. This gene is called a *prophage*, and it endows a lysogenic cell with a remarkable property, a capacity for producing virus from within itself, without the need for exogenous infection. Under normal conditions cultures of lysogenic bacteria behave just like uninfected cultures, with nearly all the cells dividing and growing at normal rates. However, occasionally, and in a random fashion, in some of the cells the prophage is "activated" and virus formation begins, leading to the formation of 200 or so bacteriophages and lysis of the cell. The fraction of cells in a lysogenic culture that make virus at any time is very small; during one division of the cells, only 1 in a hundred to one in a million will be lysed. However every bacterium in the culture possesses the genetic capacity to form virus, and this can be shown in numerous ways. For instance if certain lysogenic cultures are irradiated with ultraviolet light or X-rays, or treated with mutagenic or carcinogenic agents, the proportion of cells that can be induced to form virus reaches nearly 100% and the whole culture may disappear due to lysis; this is called induction.

Lysogenic bacteria are very abundant in nature, and their properties are identical to lysogenic cultures prepared by infection, as just described. It is probable that temperate bacteriophages are perpetuated in nature in the form of the prophage, since it is evident that a prophage in a cell might survive where a free virus would not.

The process by which bacteriophages multiply in "induced" or "activated" lysogenic cells is similar to that described

earlier in discussing the productive response. The prophage is dislodged from the chromosome. This event is followed first by multiplication of DNA units during the vegetative state, when mutation and genetic recombination can occur, and then by maturation and formation of about 200 new virus particles, leading eventually to lysis of the cell.

To summarize, lysogenic bacteria carry within themselves a genetic unit, *prophage*, which under normal conditions multiplies in harmony with the remainder of the chromosomal material and with all other bacterial constituents. Although *prophage* is not a virus, it endows a lysogenic bacterium with the capacity to produce virus from within itself, a process that occurs rarely under normal conditions and which is pathogenic to the cell. However, nearly all cells in a lysogenic population can be induced to form virus by artificial means.

The properties of prophage go beyond its ability to endow cells with virus producing capacity. The prophage can alter the genotype of a bacterial cell in other ways, one of these being by *conversion*. Many strains of C. *diphtheriae* are lysogenic and most of these are also toxigenic. Such cultures therefore produce both virus and toxin in their filtrates. Other strains of C. *diphtheriae* are *non*-lysogenic and *non*toxigenic. The nonlysogenic strain can be made lysogenic with the virus from the lysogenic strain. When this is done, it is found that the introduction of the prophage into the new strain, has converted it into a toxin producing culture. The process can best be visualized as follows: C. *diphtheriae* (lysogenic, toxigenic) yields virus which on infecting C. *diptheriae* (nonlysogenic, nontoxigenic) makes C. *diphtheriae* (lysogenic, toxigenic).

Similar changes can be effected by prophage in the somatic antigens of Salmonella. In all of these instances every cell that becomes lysogenic also acquires the new property associated with the prophage. This is not the only instance in which the genetic properties of a cell are influenced by prophage. Others include changes in phage susceptibility and in colony type. In fact it is possible that prophages perform many other functions of which we are now unaware. But the important point is that these phenomena are striking illustrations of changes in host cellular genotype effected by *prophage*, and prophage, of course, is a stage in the bacteriophage life cycle.

In view of this, it is not surprising that virus can have yet another genetic role. This phenomenon, called transduction, consists in the ability of temperate phage to transmit to the genotype of a recipient cell by infection some of the properties of the cell in which it was last grown. Unlike *conversion*, where all cells which incorporate the prophage *necessarily* become altered in *one* genetic property, in transduction some of the cells which incorporate the prophage do not necessarily become altered in a recognizable way, and in addition individual cells in a culture may be altered in different ways. It appears as though the virus incorporates within its own structure some of the DNA of the cell in which it is grown and it seems to do this in a nonselective way so that a virus multiplying in one cell will pick up one set of genetic properties and other viruses in other cells will incorporate other parts of the DNA. When a new lysogenic cell is now formed by infection with these bacteriophage DNA's, some of the DNA from the first set of bacteria becomes integrated into the DNA of the second bacteria. The net effect of this transduction process is a transfer of some of the properties of one bacterium to another. It is evident how important such a process might be for the general genetic evolution of bacteria.

In summary, bacterial viruses can be visualized as organisms capable of re-

production either, as prophage, where they are completely integrated with the bacterial genome and multiply in unison with it, or as vegetative units, where they can undergo genetic variation and recombination, and where the final result is more virus particles and destruction of the bacterium. In addition, the virus particles can act as vectors for the transmission of genetic material from cell to cell.

The physiology, biochemistry and genetics of animal, plant, and insect viruses is less understood than that of the bacteriophages, for at least three reasons. Understandably, with such viruses, the emphasis in research has been on obtaining information about pathogenesis of the virus to the organism, rather than to the cell, and studies of virus multiplication in a whole organism are obviously fraught with complications. Secondly, success with the bacteriophages is largely due to the availability of a suitable host cell which can be grown easily, under reproducible conditions. Until recently this has been difficult to do with the animal viruses, and there are still difficulties with the plant and insect viruses. Finally, this latter group of viruses may be inherently more complex.

Nevertheless, the study of animal virology is proceeding rapidly. In general features, the multiplication of viruses in animal cells is similar to that of the bacteriophages in bacteria. One growth cycle again involves infection (with mutation and genetic recombination), eclipse phase, maturation and release of virus. However certain differences from the bacterial viruses are already apparent. Whereas bacterial viruses contain protein and only DNA, animal viruses contain protein and *either* DNA or RNA, or both, and possibly some lipids. With some animal viruses, release of the virus is not by lysis but by secretion. There is as yet no clear knowledge of how the earlier stages of infection are initiated with the animal viruses, by injection, or by entrance of the whole particle, or by some other mechanism.

Lysogeny has never yet been clearly demonstrated with the animal viruses, although many instances of viruses grow-in some sort of symbiotic union with cells have been observed. Studies on these latter systems may serve as guides to an understanding of latent viruses in animals, such as the herpes virus system in man.

The fact that the nucleic acid in some of the animal viruses is RNA and not DNA is of considerable interest from the genetic point of view. For many years mainly from work with microorganisms and bacteriophage it was felt that the material in the cell which controlled genetic properties was DNA. However no DNA has been found in some of the animal viruses which have been extensively purified. The genetic continuity in such viruses must therefore be due to either RNA or protein, and as we shall see presently, it is probably the RNA which is the active unit.

In bacteriophage, the separation of nucleic acid and protein occurs on infection. A similar separation of nucleic acid and protein can also be effected with some of the animal viruses, and with a plant virus, tobacco mosaic virus. These latter viruses which contain only protein and RNA can be treated chemically when they are in the resting state outside the cell, so that preparations consisting almost completely of RNA are obtained. These RNA preparations when added to host cells, will multiply and produce complete virus progeny, containing both protein and RNA, so that information required for the production of virus protein and progeny particles is carried into the cell by RNA alone.

This indicates that either nucleic acid, RNA or DNA, may play the role of active unit in viruses, and that the protein probably acts only as a protective carrier for

nucleic acid. This is more complicated than the simple picture one had a few years ago of DNA being the only possible chemical substance involved in heredity. However there is possibly a close relationship between the multiplication of RNA and DNA, and this whole question is under extensive investigation at the present time.

This article up to now has not concerned itself with the relationship of viruses to disease. But there are certain recent developments in the aetiological role of viruses in tumour induction which merit a few words, particularly since they point out the use and the need for the fundamental virus research discussed earlier.

Viruses have been shown to be involved in the induction of tumors in mice. In addition it has been found that normal mouse tissues, or tumors induced by X-rays, contain tumor virus. These results raise a number of questions about the relation of mammalian viruses to cells, and about how mammalian virus can change the genotype of a cell so that it can become a tumor cell. It may well be that the bacterial systems discussed earlier are prototypes for the mammalian systems. Normal mouse cells may carry a *provirus* or a virus in some sort of latent state which can be uncovered or "induced" by various agents. Also tumor virus like temperate virus may on infection of a mammalian cell, be integrated in some form or another, and change the cell into a tumor cell. These are only working models, and it is just as conceivable that the relationship of tumor virus to cell may involve a new kind of virus cell interaction of which we are at present unaware. However, it is clear that viruses play a central role both in disease and in fundamental studies in biology and many challenging and exciting problems await research workers in this field.

ELECTRON MICROSCOPY OF VIRUS TUMORS IN MICE

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THE development during the past few years of satisfactory methods of preparing and sectioning tissue for study in the electron microscope has given increased impetus to the investigation of viruses and virus-cell interaction with the aid of this instrument. Concurrently with these developments, the successful induction in several laboratories of many kinds of tumors in mice with cell-free extracts has aroused considerable interest in the role of viruses in the etiology of cancer. In this article some recent findings obtained by applying the electron microscope to the study of virus tumors in mice will be described and illustrated with examples drawn from work carried out in our laboratory.*

The first virus known to be associated with tumors in mice was the mammary tumor agent (1). In certain strains of mice, notably the C3H strain, the virus

*For more general discussions of the electron microscopy of tumor viruses and for full references the reader is referred to the following articles:

(1) Bernhard, W.: Electron Microscopy of Tumor Cells and Tumor Viruses. A Review. Cancer Research, *18*:491, 1958.

(2) Dmochowski, L., and Grey, C. E.: Subcellular Structures of Possible Viral Origin in Some Mammalian Tumors. Ann. N.Y. Acad. Sc., *68*:559, 1957.

is present in the milk of lactating females. The virus, imbibed by the offspring, has no immediate apparent effect, but many months later, and especially if the mammary glands of these mice have been stimulated by hormones or by successive periods of lactation, many of them succumb to mammary tumors from which virus can be extracted.

Examination in the electron microscope of sections of mammary tumors from these mice almost always reveals characteristic bodies within the cytoplasm of the cells and in the spaces between cells. Two types of particle may be distinguished, one intracellular and the other extracellular. The intracellular particles vary greatly in number from one tumor to another and also in different regions of the same tumor. They may occur in small scattered groups within the cytoplasm or they may form large dense aggregates as illustrated in Fig. 1. These aggregates occupy a characteristic juxtanuclear position, a site associated in the normal cell with the Golgi apparatus. In some tumors the aggregates are large enough to be visible as distinct inclusions with the light microscope.

Examination of the intracellular particles at high power in the electron microscope reveals that each consists of a round membrane-bound body of uniform density and of diameter about 75 mμ. The limiting membrane is double-layered, giving the particles a doughnut-like appearance as shown in Fig. 2. The extracellular form is distinctly different from the intracellular, being larger and more variable in size. The average diameter is about 110 mμ. Many particles of the extracellular type contain a small, dense, usually eccentric body called a nucleoid. Fig. 3 shows several examples in a lumen close to the surface of a cell. The less dense and irregularly shaped structures seen in association with the virus particles are sections through the microvilli which project from the cell surface. Particles are frequently seen at the ends of microvilli, suggesting that passage through these finger-like projections may be the mode of release of the viruses from the cell.† Appearances suggestive of transformation stages between the intracellular and extracellular forms may be seen in particles within microvilli or close to the cell surface.

The size of the particles observed in these tumors is well within the size-range of known animal viruses, and the general appearance described—a membrane-bound body containing a dense nucleoid—is one that has come to be associated with many animal viruses as seen in the electron microscope. Such well known viruses as, for example, vaccinia or herpes simplex have a similar appearance at certain stages of development. Moreover, in normal tissues such bodies are rarely if ever seen. It is therefore reasonable to describe the bodies observed in mouse mammary tumors as viruses, and it is tempting to identify them with the mammary tumor agent. However such an identification is by no means proved by observations such as those described. One fact that immediately poses a problem is the finding of bodies, apparently identical to those described, in mammary tumors in mice of the C3Hf subline (Fig. 4). Mice of the C3Hf subline are genetically the same as C3H mice but they are descended from C3H mice which had been foster-nursed immediately after birth by mothers of a strain known to be free of the mammary tumor agent. Thus if the agent is carried to the offspring only in the milk, C3Hf mice should be free of the agent and should have a low incidence of mammary

†Care must be taken in making this interpretation since some of these appearances are due to abrupt changes in the direction of the microvilli as they pass out of the section.

tumors. The incidence is indeed much lower than in C3H mice but mammary tumors do occur in C3Hf mice and, as mentioned above, some of these contain particles identical with those seen in agent-containing mice. The reasons for this apparently anomalous finding are not yet clear but possible explanations include the following:

(1) Some C3Hf mice may carry the mammary tumor agent, having been infected by some unknown route.

(2) The particles observed in the electron microscope both in C3H and C3Hf tumors may not be the tumor virus but some other virus that is carried in the mice and grows preferentially in the tumor cells.

(3) The particles may represent a pathological secretion of the tumor cells rather than virus. The close association with the Golgi region lends credence to this view since the Golgi region is intimately associated with secretion in many cells.

The solution of the problem of identification awaits the development of better methods of titrating the virus and of preparing purified virus extracts. Given these conditions it should be possible to obtain a correlation between the number of particles in an extract similar to those seen in electron micrographs of tumor cells and the biological potency of that extract. Only then would the identity of the presumed virus particles with the mammary tumor agent be unequivocally established.

The presence of virus-like particles in C3H mice is not, however, confined to mammary tumors. In two other kinds of tumors in C3H mice studied in our laboratory, virus-like particles have been observed in large numbers. The first of these was a parotid tumor which arose in a mouse that, in connection with another experiment, had been exposed eight months previously to 800 r of X-radiation and then injected intravenously with rat bone marrow cells to prevent radiation death. Cells of this tumor contained numerous vesicular or 'doughnut' particles and also a few particles with dense central bodies (Fig. 4). In size and appearance these resemble closely the particles encountered in mammary tumors.

The second was a transplantable plasma cell tumor (2, 3, 4) which arose originally in a C3H mouse at the Cancer Research Genetics Laboratory, Berkeley, California, and which has been maintained for over eighteen months in our laboratory by serial transfer in C3Hf mice. Cells of this tumor regularly exhibit large inclusion bodies which on examination in the electron microscope are seen to consist of large numbers of vesicular particles with double-layered limiting membranes, apparently identical with the intracellular form present in mammary tumors of C3H mice. Fig. 5 shows a low magnification picture of a cell from an ascitic form of the tumor. Most of the particles occur in a dense aggregate immediately above the nucleus but a few may be seen within the ergastoplasmic vesicles especially to the left of the nucleus. Fig. 7 is a higher magnification picture in which the individual particles may be seen and compared with those in mammary tumors (Fig. 2). A close connection between the Golgi apparatus and the presumed virus particles is again evident in these cells, and, in fact, transition forms between apparently normal Golgi microvesicles and typical virus particles are often seen. No extracellular particles with dense centres or nucleoids have been observed in the plasma cell tumors.

The significance of these observations remains to be elucidated. One important objective is to determine whether tumor inducing agents can be extracted from these tumors. Fortunately, methods of testing tumor extracts for carcinogenic effect have been greatly improved during the past few years by two discoveries,

one due to Gross (5, 6), the other to Stewart and her collaborators (7).

Gross was the first to demonstrate clearly the importance of using newborn animals in testing tumor-virus extracts for potency. He showed that cell-free extracts of organs from leukemic mice would, after an interval of several months, produce leukemia and also solid tumors if injected into newborn C3H mice, but not if injected into older animals.

Mice injected at birth with cell-free extract of leukemic tissues were sent to us by Dr. Gross when symptoms of their developing leukemia became evident. Tissues such as thymus and lymph node from these mice showed virus-like particles which were not seen in corresponding normal tissues. The particles occur extra-cellularly, usually in small groups in spaces between cells (Fig. 8), but also occasionally in large vesicles within cells. They are about the same size as the particles found extracellularly in mammary tumors but differ from them in having a larger and more centrally placed nucleoid. Particles of the vesicular or "doughnut" type which are so prominent in mammary tumors are rarely found in these tumors. Here again, although the presence of typical virus-like bodies in virus-induced tumors is very interesting and suggestive, conclusive proof of their identity with the tumor-inducing agent is lacking.

The susceptibility of newborn mice to infection by tumor viruses may well be due to their immunity mechanism being insufficiently developed at birth. In the very young animal the virus may gain a foothold before antibody production is established. Another situation favourable to virus multiplication occurs when the virus is introduced into cells growing outside the body in tissue cultures. In 1957 Stewart and her collaborators reported important experiments in which cell-free extracts prepared from leukemic mice were introduced into cultures of cells. After several serial passages in cells growing *in vitro* the fluid from the cultures was injected into newborn mice. A high percentage of these mice subsequently developed tumors of many different kinds. It was shown in these and subsequent experiments that not only was the range of tumor types induced by the agent increased by passage in tissue culture, but its sphere of activity was extended to other strains of mice and even to other species. Thus, allowing the virus to multiply in cultivated cells apparently increased its virulence either by changing the properties of the virus itself or by causing a particularly virulent strain to emerge.

We have examined in the electron microscope tumors induced in Swiss mice in our laboratory by an extract obtained from Drs. Stewart and Eddy. In these, virus-like particles were at first difficult to find, but after some experience in searching was gained, it was possible in some tumors to detect bodies which stood out because of their uniform size and high density. These occurred usually in spaces between cells, either singly or in small groups. Closer inspection shows that the bodies have the characteristics of virus particles, namely a dense central body surrounded by a less dense zone and bounded by a dense membrane (Fig. 9). The particles resemble those seen in tumors induced by the Gross virus (Fig. 8) rather than those present in C3H mammary tumors (Fig. 3), in that the nucleoid is relatively large and centrally placed. Intracellular forms have not been definitely identified.

The small number of virus-like particles seen in the highly malignant tumors induced by the Stewart-Eddy virus is at first sight surprising. It is, however, in accord with more general observations we have made on the relation of the number of virus particles seen in a tumor to the degree of differentiation of the cells.

In our experience highly differentiated tumors with well-defined acinar structures (see for example Figs. 1, 4, 10) contain in general many more viruses than undifferentiated anaplastic tumors. This observation is in accord with the experience of Gross who finds it more difficult to extract virus from highly malignant tumors than from less malignant ones. A possible explanation is that the virus initiates tumor growth, but after this is established virus is no longer required to maintain continuing growth. Virus production by the cells would then be secondary to growth and division of the cells, and perhaps in rapidly growing malignant tumors, cell growth eventually outstrips virus growth. Another possibility is that virus is present in tumor cells in a form that has not yet been recognized in the electron microscope.

In order to investigate the generality of Stewart and Eddy's method of obtaining potent virus extracts, Dr. McCulloch of our laboratory used as starting material a mammary tumor (8). Figs. 1–3 illustrate the appearance of cells from the actual tumor used. An extract of the tumor was introduced into a culture of mouse kidney cells. After about two weeks the culture degenerated and fluid from it was added to a fresh culture of cells. After four days a cytopathogenic effect (C.P.E.) was observed, and the procedure was repeated on a fresh culture. Thereafter a C.P.E. was regularly obtained three or four days after inoculation into fresh cultures. Fluid from the fifth passage was injected into newborn Swiss mice. After an interval of about four months the first tumor was observed, and to date about 40% of the injected mice have succumbed to tumors of three main types—parotid and mammary carcinomas and thymic lymphomas.

Ten of the induced tumors have been examined with the electron microscope. In two of these, one a mammary and the other a parotid tumor, large numbers of virus-like particles were observed within the lumina of acini. Fig. 10 shows a typical field in a section from the parotid tumor. Most of the particles have a large centrally-placed nucleoid (shown more clearly in Fig. 11) and have a fairly uniform diameter of about 110mμ. Thus they resemble the particles seen in tumors induced by the S.E. virus and the Gross virus more than those in the tumor of origin. The particles present in the induced mammary tumor (Fig. 12) are apparently identical with those in the parotid tumor. Intracellular vesicular bodies of the type seen in the original mammary tumor were not observed, but in the parotid tumor larger bodies of variable size and uniform density were observed within the cytoplasm (Fig. 10, upper part). The significance of these bodies is not known at present.

Virus-like particles similar to those shown in Figs. 10–12 were seen in smaller numbers in four other induced tumors. In the remainder virus-like particles have not yet been identified but further search may well be required to detect them if they are present in very small numbers. In these tumors a correlation between the degree of differentiation and the number of virus-like particles present was again noted.

Many questions remain to be answered. For example, what part, if any, does virus play in tumors induced by radiation or chemical carcinogens? What is the mechanism of tumor induction by viruses; is its action on the cells direct or indirect? Is virus necessary for maintaining growth after the tumor is started? What is the significance of the newborn state in the induction of tumors? The answer to these and many other questions can now be sought by applying new techniques that have recently become available. An important contribution to the solution of these problems will certainly be made by the electron microscope.

Acknowledgments

It is not possible to acknowledge in detail the numerous sources of information used in writing this article, nor the valuable contributions of many colleagues. Special mention should be made, however, of Mrs. June D. Almeida who cooperated closely in the electron microscope work and was responsible for many of the micrographs reproduced here.

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TUMOR-HOST RELATIONS: A REVIEW

E. S. GORANSON

THE genesis of cancer involves the acquisition of properties enabling cells to proliferate at the expense of, and ultimately with the death of the host organism. Malignant cells are characterized by the loss of intrinsic factors regulating cellular growth and differentiation (genetic), and also by a release from extrinsic, or host, factors which govern metabolism and growth (humoral). However, the concept that cancerous growth is an autonomous process is true in a relative sense only since tumors may be influenced by the hormonal status and possibly by the specific defense mechanisms of the host. The expression "tumor-host relations" therefore implies a reciprocal influence although the dominant aspect of this relationship is without question the impact of the tumor on its host.

The purpose of this discussion is to outline some general systemic effects of tumors (apart from tumors directly involving endocrine organs where the systemic effects may be attributable in a large measure to excess hormone production), to indicate inferences that may be drawn as to their significance and to consider mechanisms suggested as underlying these effects. For a more detailed and comprehensive treatment of this subject the reader is referred to excellent reviews by Fenninger and Mider ('54), Noble ('57), Homburger ('57), Begg ('58), Nakahara and Fukuoka ('58), and Greenstein ('54).

In investigating tumor influences on the host the experimentalist has advantages denied the clinician. He has a wide choice as to the mode of tumor induction (the application of carcinogens, the injection of tumor viruses, the selection of animal strains with high incidences of spontaneous tumors, and transplantation), he can follow the course of tumorigenesis and chemically evaluate stages in this process, and he has adequate controls. In establishing an effect as tumor-induced the experimental criteria are those employed by the endocrinologist in determining whether or not an organ possesses an endocrine function. First it is necessary to show that the effect occurs at a site or at sites in the animal remote from the tumor, and bears some quantitative relationship to tumor size; second, to show that the effect subsides following

removal or regression of the tumor. The majority of studies in this field have been carried out using transplantable tumors which is perhaps unfortunate from the standpoint of attempting to correlate some experimental results with clinical findings.

Systemic Effects of Tumors

One generalization that has emerged from tumor-host studies is that not only do tumors arising in various organs present a fairly uniform set of biochemical properties, but tumors also produce more or less uniform effects on the carrier organism.

Various investigators describe *three* phases in the growth of a transplanted tumor with respect to its effects on the host. These are well illustrated in careful metabolic studies by Mider and his group ('49) using subcutaneous transplants of the Walker 256 adenocarcinoma in the rat. The first phase is characterized by the absence of major systemic effects. During this period of 6–10 days after implantation both tumor and host tissues show weight gains and the animal remains in nitrogen balance. The second phase, of approximately 10 days, is distinguished by an acceleration in tumor growth and a loss of carcass weight (host minus tumor). In the third or preterminal phase, of 1 or 2 days duration, the host tissues show rapid wasting (cachexia), there is a marked increase in nitrogen excretion and tumor growth may cease.

Weight loss during the second phase, which occurs when tumors attain a critical size (5 to 10% of body weight) is attributed to three factors: (a) diminished food intake or anorexia, (b) translocation of nitrogen from carcass protein to the tumor, and (c) loss of stored fat.

When the dietary source of nitrogen becomes inadequate the resources of the host are drawn upon. This is established from the nitrogen balance and metabolic studies of Mider ('49) and also by the isotope studies of Heidelberger ('54), Le Page ('52) and Greenberg ('51). These investigations support the concept advanced by Mider that the rapidly growing tumor serves as a "nitrogen trap."

In mice maintained on a low protein diet and in negative nitrogen balance, some retardation in tumor growth does occur, (White, 1945). A negative nitrogen balance induced by the injection of cortisone slows the progress of a tumor (Ingle, 1950). Treatment with glucagon, which causes an anorexia and also stimulates protein catabolism, has been shown to have an inhibitory effect on the growth of Walker tumor transplants in the rat (Salter, Meyer and Best 1958). This effect of glucagon has been confirmed in the rat bearing implants of the Novikoff hepatoma and in DBA mice with an ascites tumor (Goranson, in press).

A somewhat different approach to this problem has been the study of the influence on tumors of diabetes, a condition in which the disturbance in protein metabolism might be expected to impose metabolic restriction on neoplastic growth. Retardation in the growth of tumors in diabetic animals has been reported by a number of investigators (Goranson et al. 1954; McKee et al. 1955; Ingle, 1958; Gillman et al. 1958; Klärner and Klärner, 1958). One possible explanation is that a division of available protein between the "competitive" processes of gluconeogenesis on the one hand and tumor growth on the other may exert a limiting influence on tumor growth.

The decrease in the total lipid content in the tumor-bearing rat is much greater than that of a control animal on the same restricted diet. The mobilization of depot fat is reflected by a hyperlipemia, which appears in the first phase, reaches a maximum in the second phase and falls off

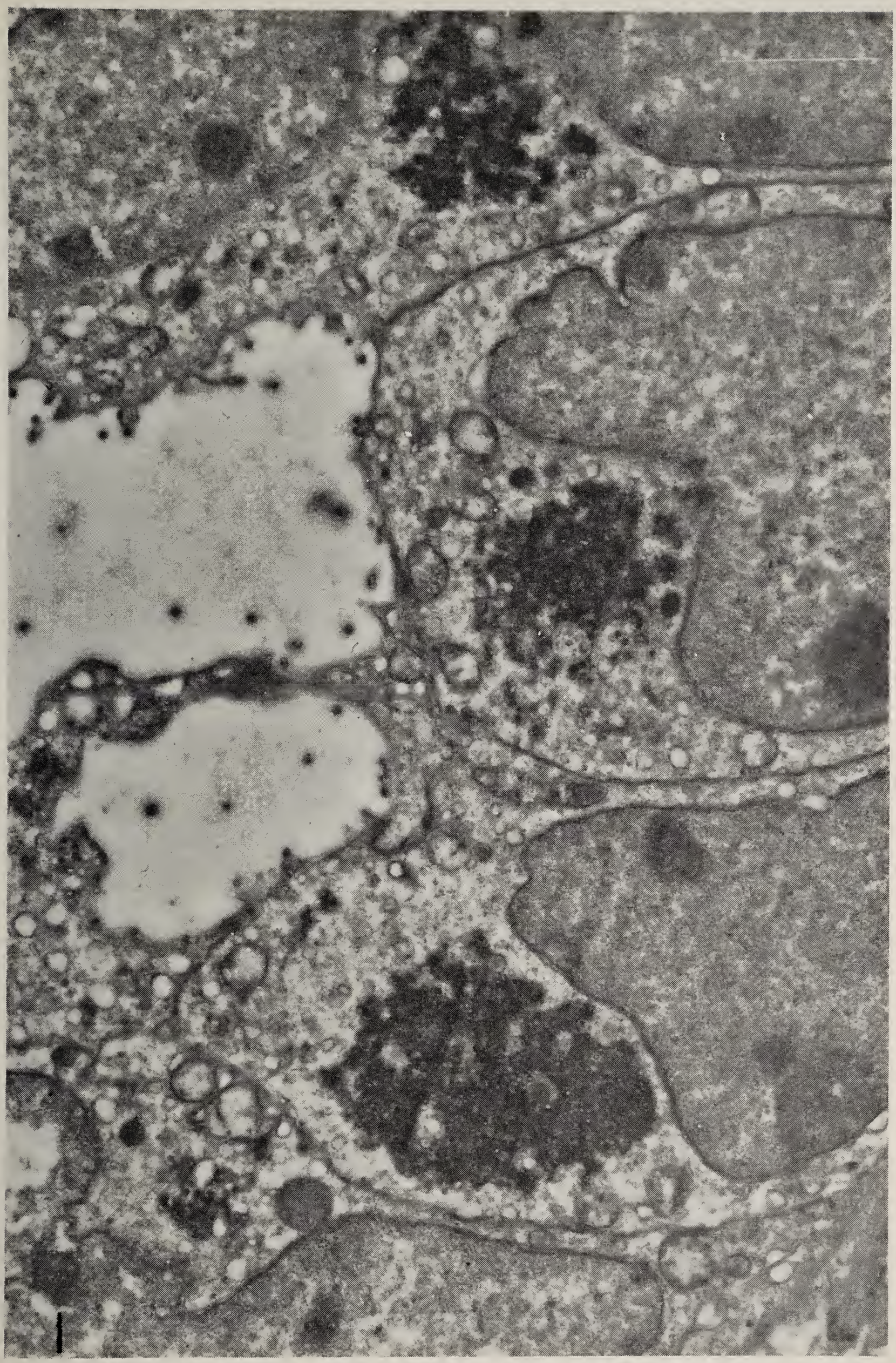
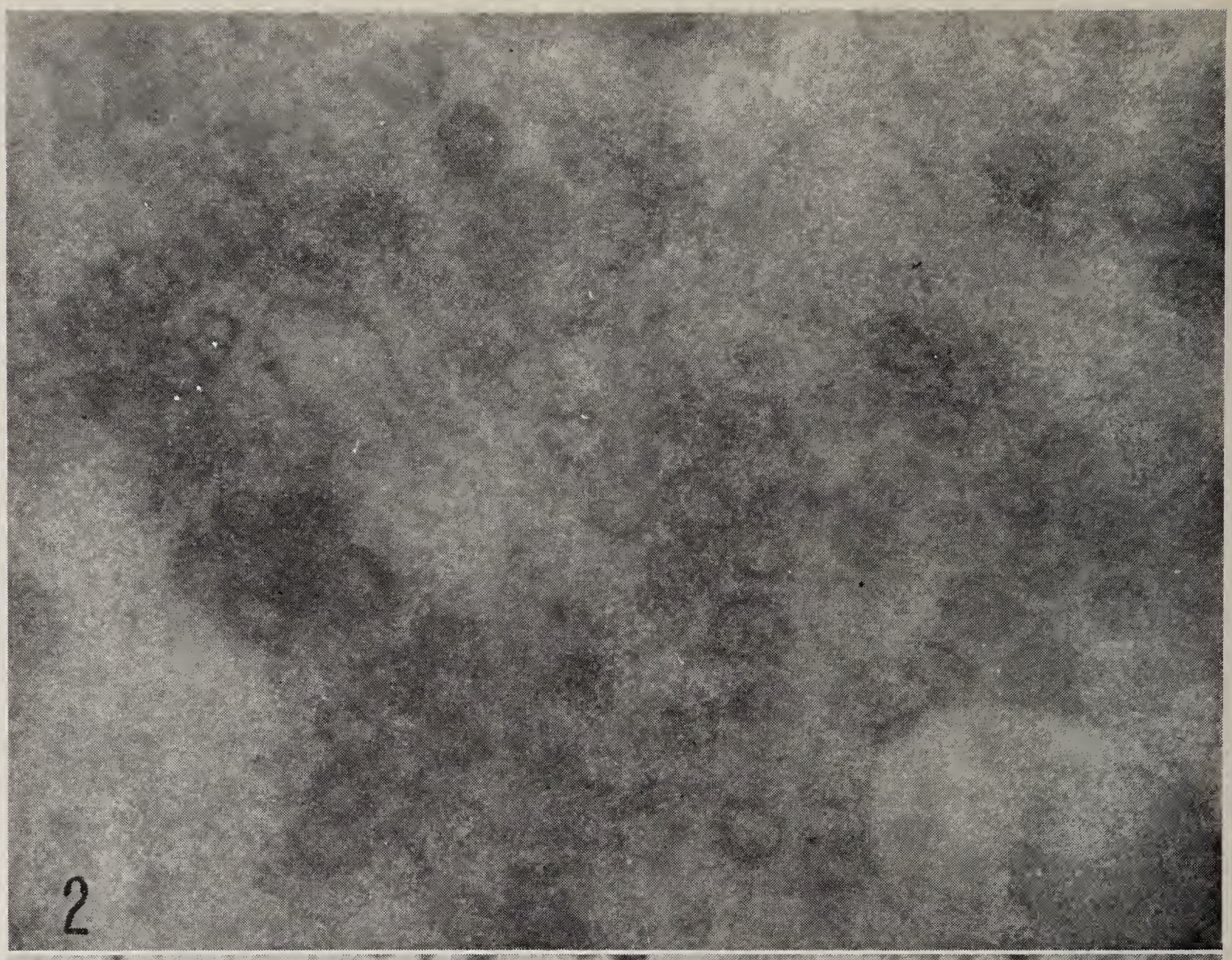


FIGURE 1. Mammary tumor in C3H mouse. The micrograph shows dense juxtanuclear inclusions made up of masses of densely packed vesicular bodies. In the lumen (middle left) a few extracellular forms may be seen. 13,000 X



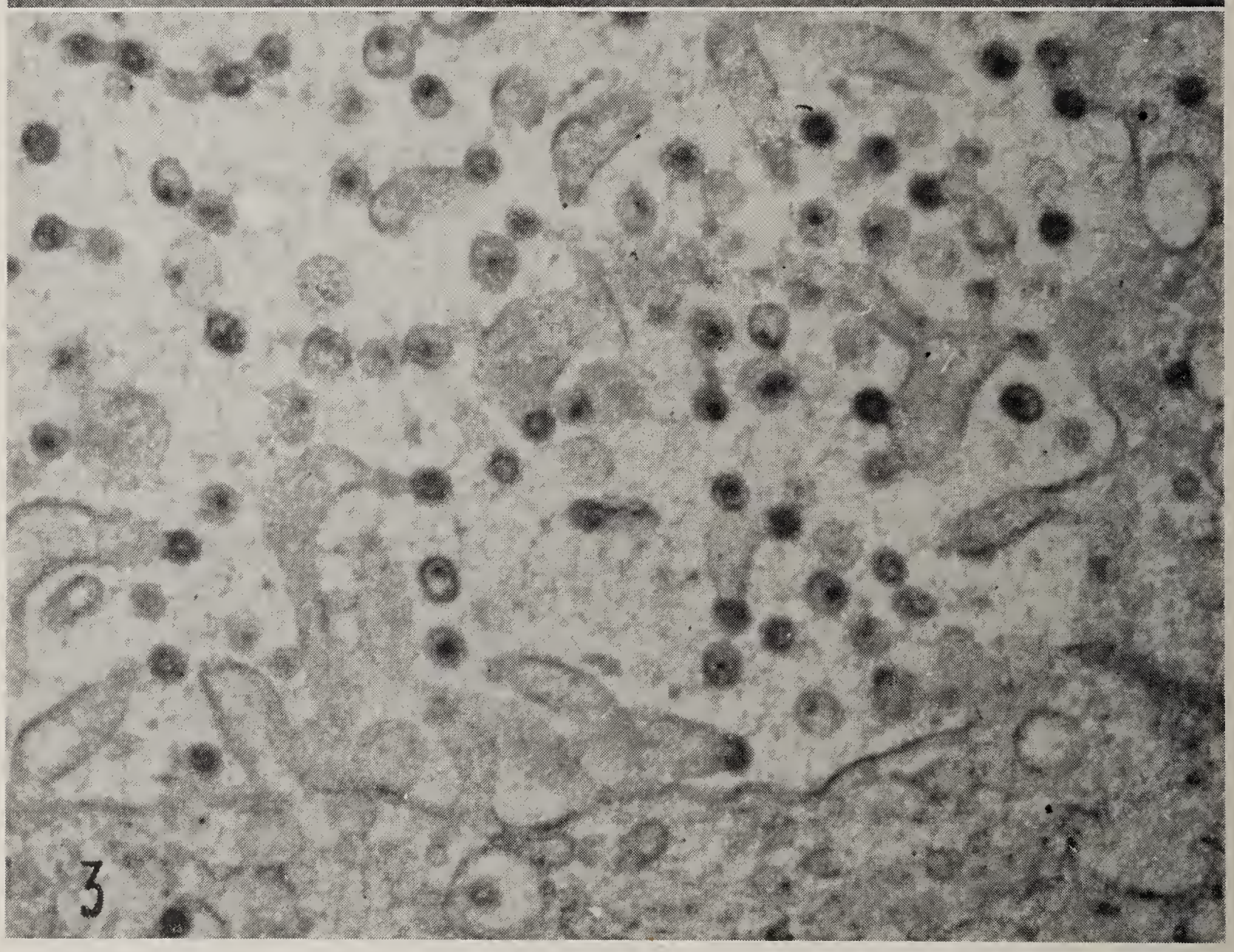
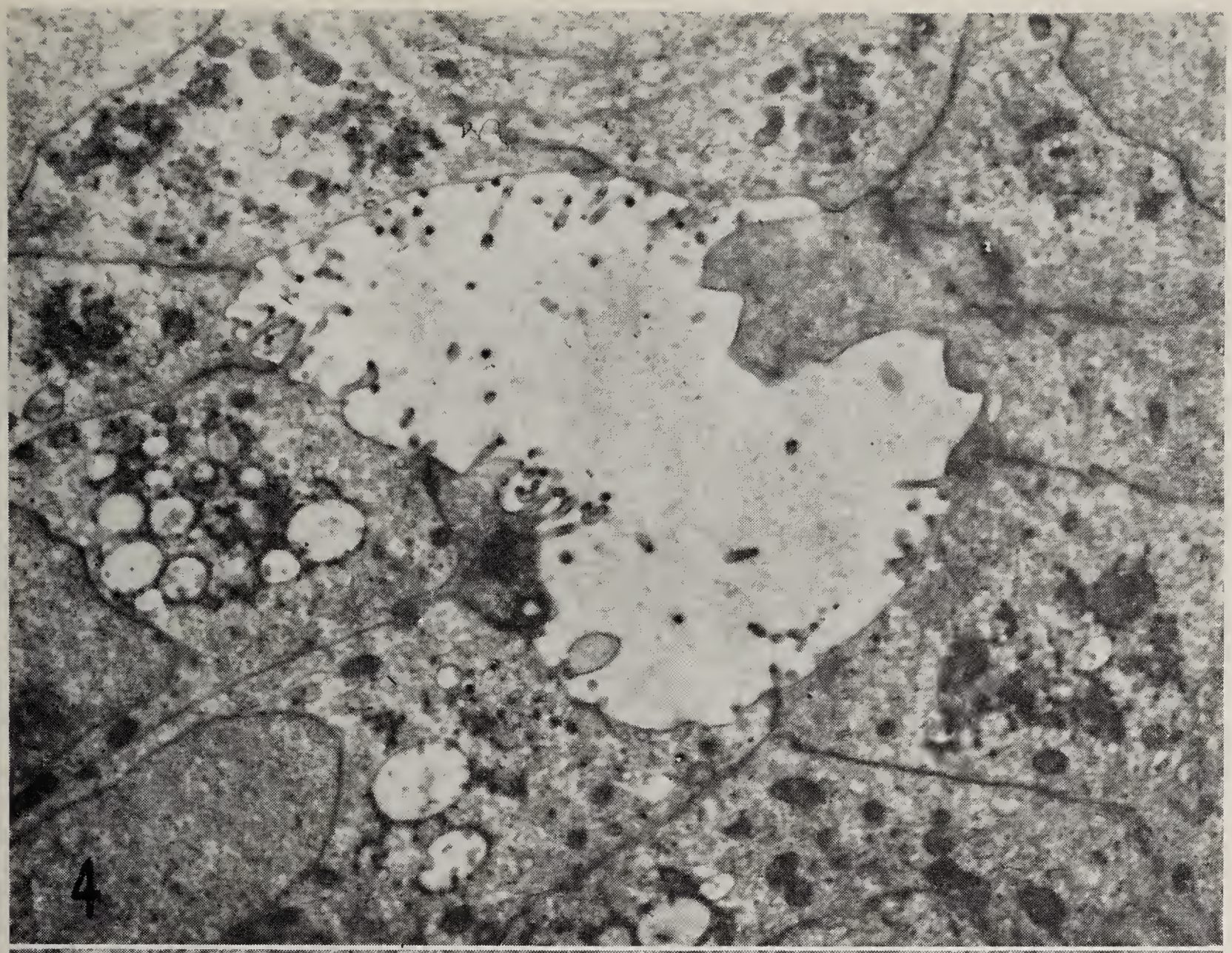


FIGURE 2. A small portion of an inclusion similar to those in Fig. 1 at high magnification, showing the structure of individual particles. The double-layered membrane surrounding each particle is clearly visible. 115,000 X

FIGURE 3. An area near the surface of a cell from a C3H mammary tumor showing an irregular cell border with many projecting microvilli. In and around the microvilli are numerous virus-like particles, many containing small dense nucleoids. 38,000 X



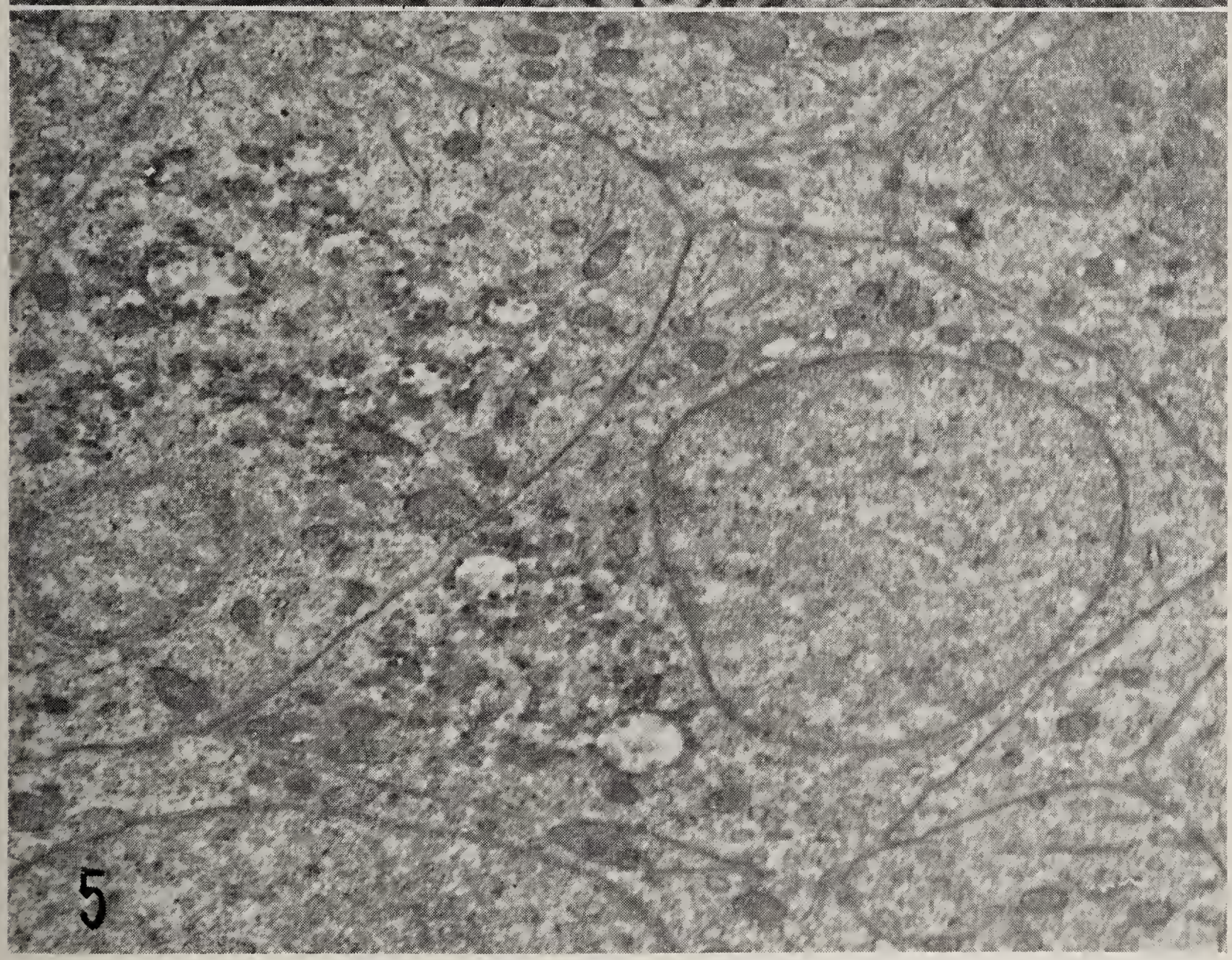
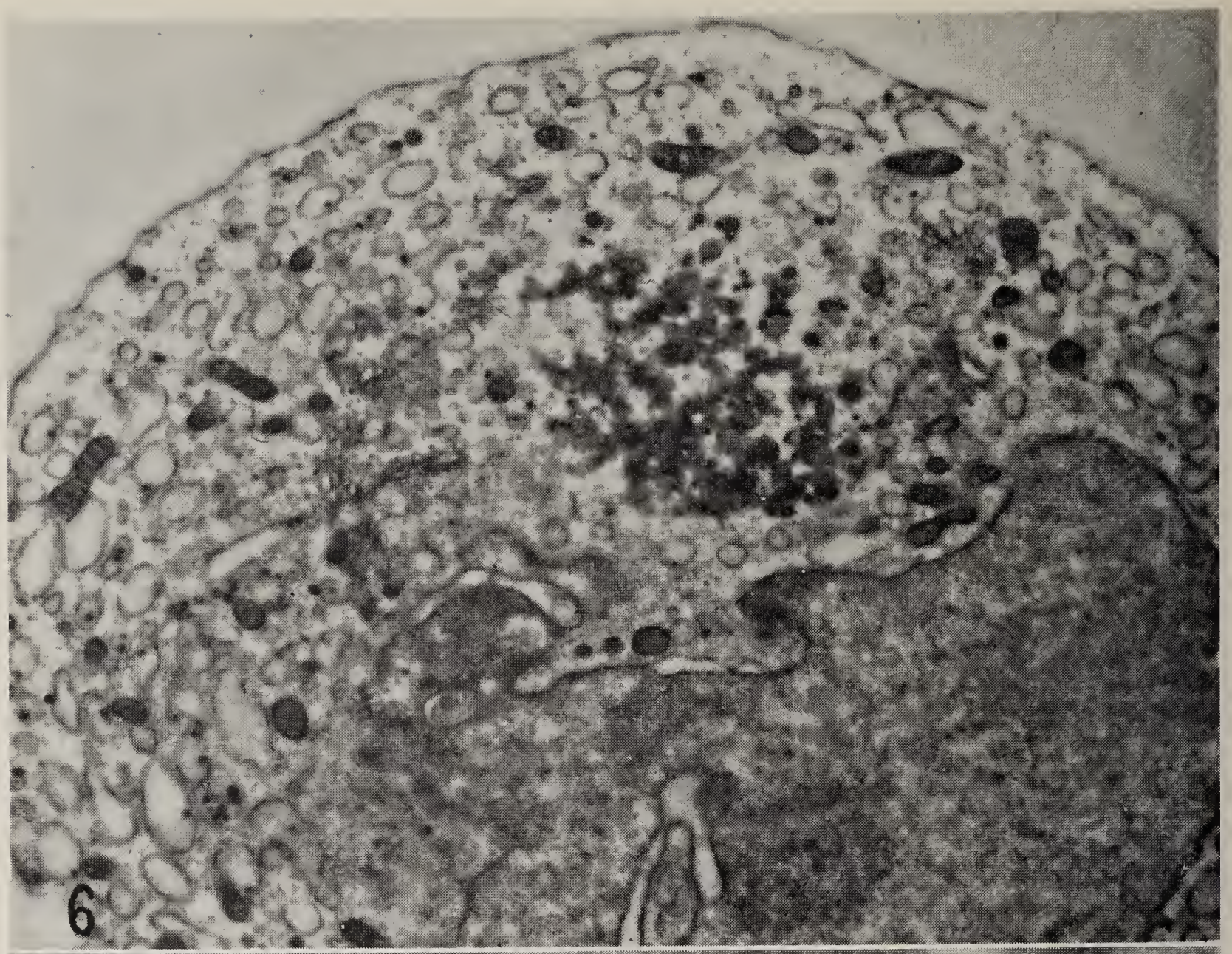


FIGURE 4. Mammary tumor in a C3Hf mouse, showing typical intracellular and extracellular virus-like forms in a tumor from a supposedly agent-free mouse. 8,000 X

FIGURE 5. Parotid tumor induced by X-radiation in a C3H mouse. The cells contain many virus-like particles similar to those observed in C3H mammary tumors. 14,500 X



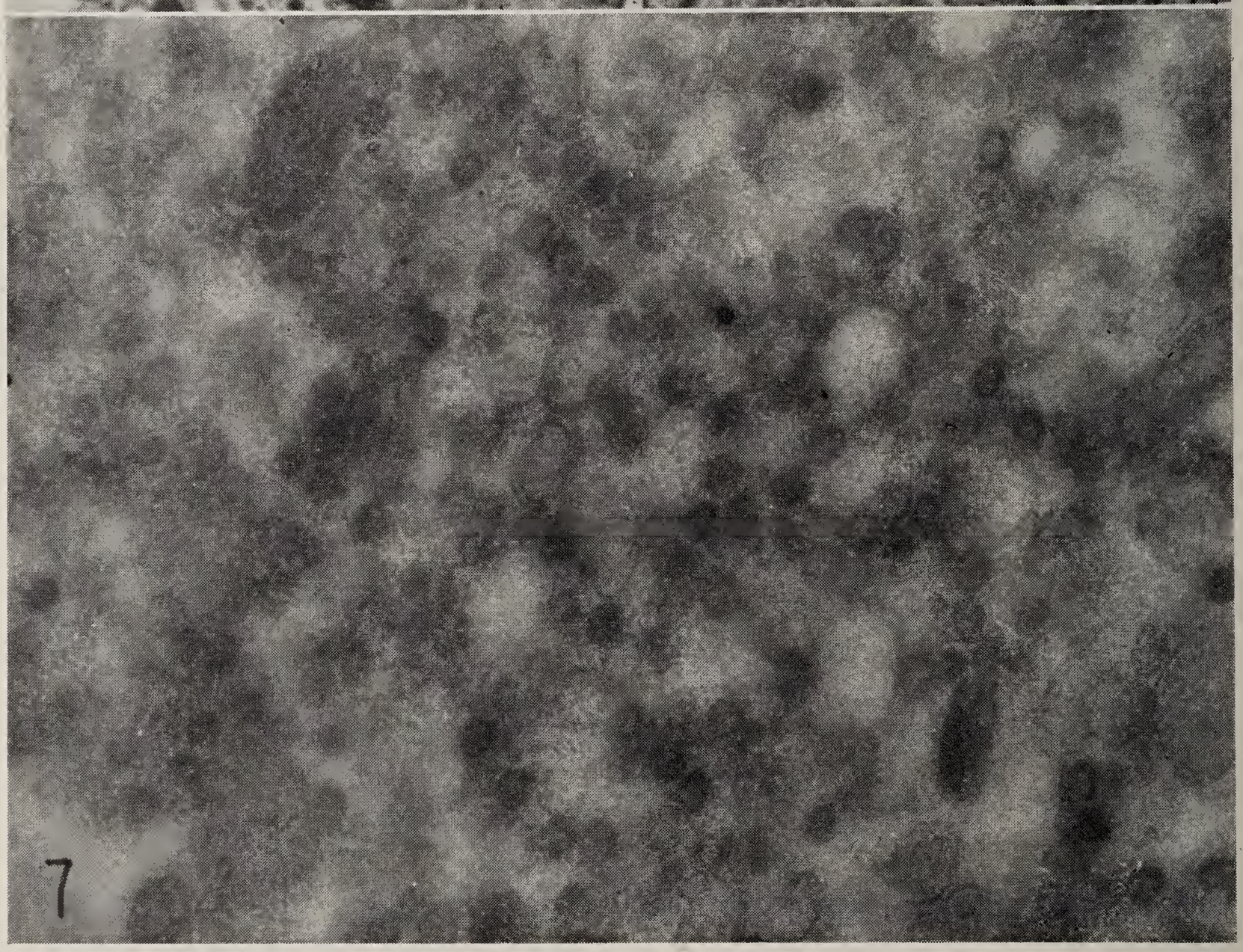
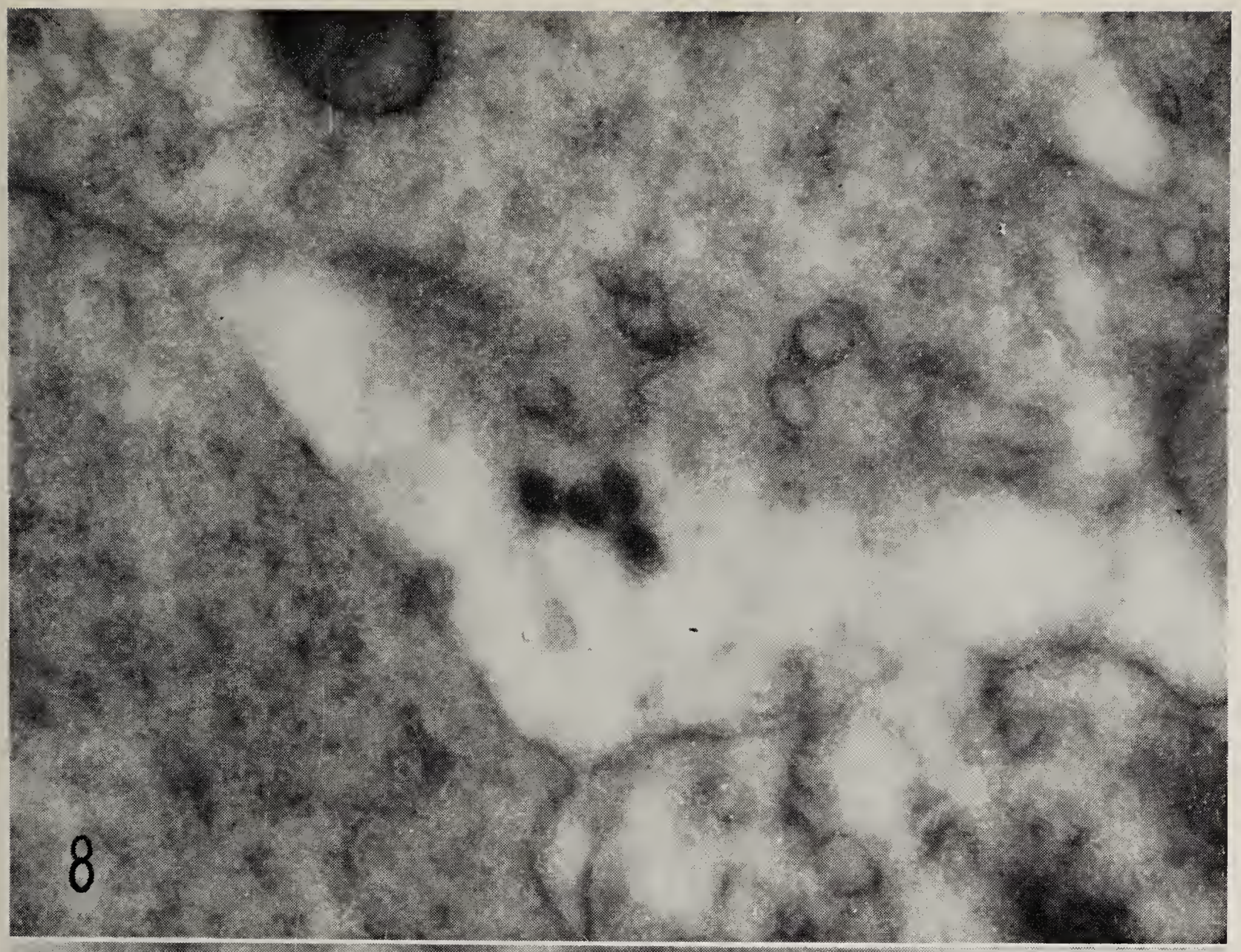


FIGURE 6. Plasma cell tumor showing a cell from an ascitic form. Above the irregularly-shaped nucleus there is a dense inclusion which at higher magnification is seen to consist of virus-like particles interspersed with larger bodies of irregular size and uniform density. Golgi vesicles of normal appearance are visible to left and right of the inclusion. 11,000 X

FIGURE 7. Plasma cell tumor. High magnification micrograph of part of an inclusion showing individual particles of "doughnut" form similar to the intracellular form found in mammary tumors (Fig. 2). A few of the larger bodies mentioned in the previous legend can also be seen. 80,000 X



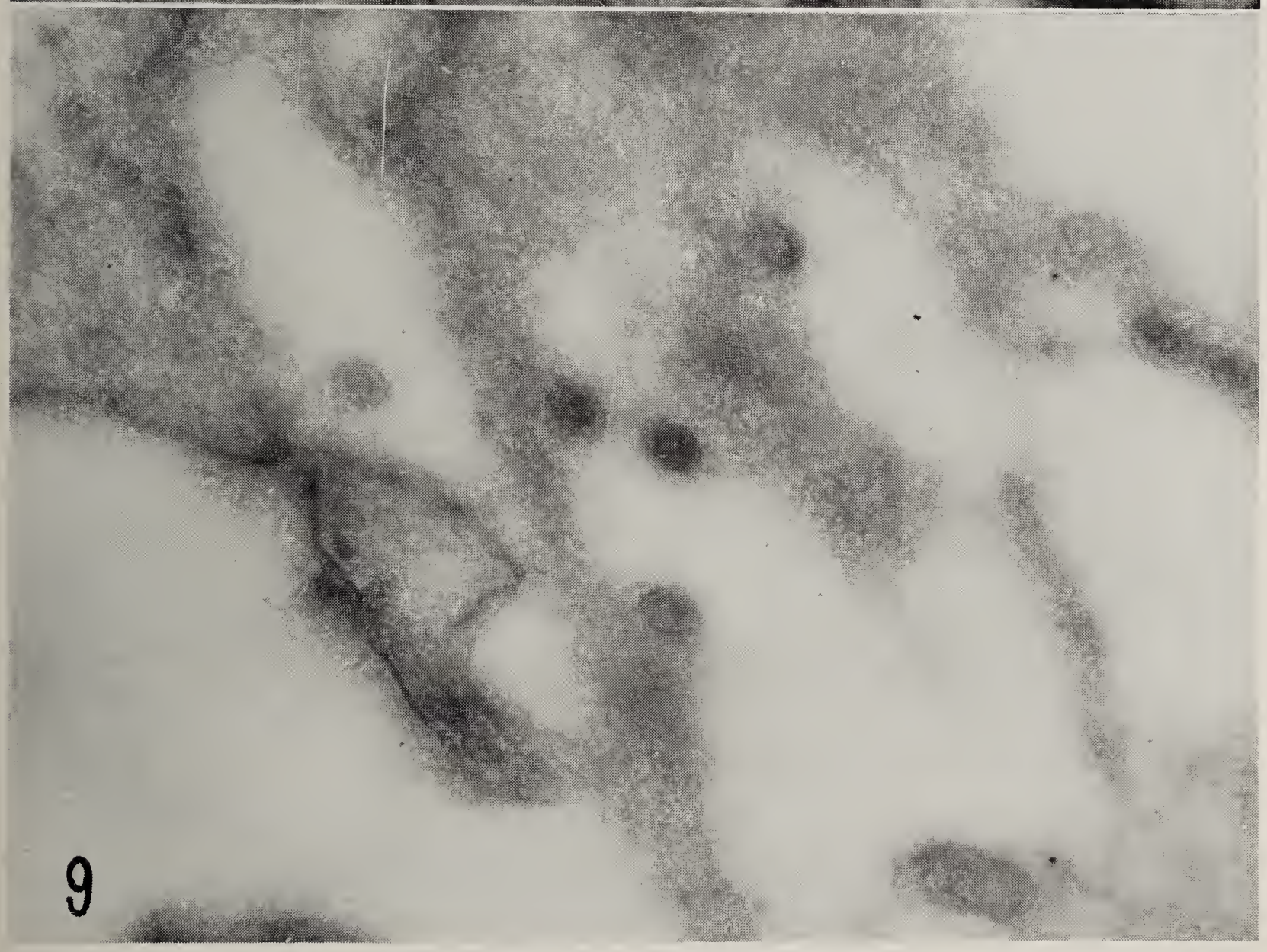


FIGURE 8. Thymus from C3H mouse with leukemia induced by Gross extract. Small groups of extracellular virus-like particles such as those in the centre of this picture were encountered in leukemic tissues of the mouse. 50,000 X

FIGURE 9. Mammary tumor induced in Swiss mouse by S.E. extract. Sparsely distributed extracellular virus-like particles such as those illustrated were found throughout the tumor. 51,000 X

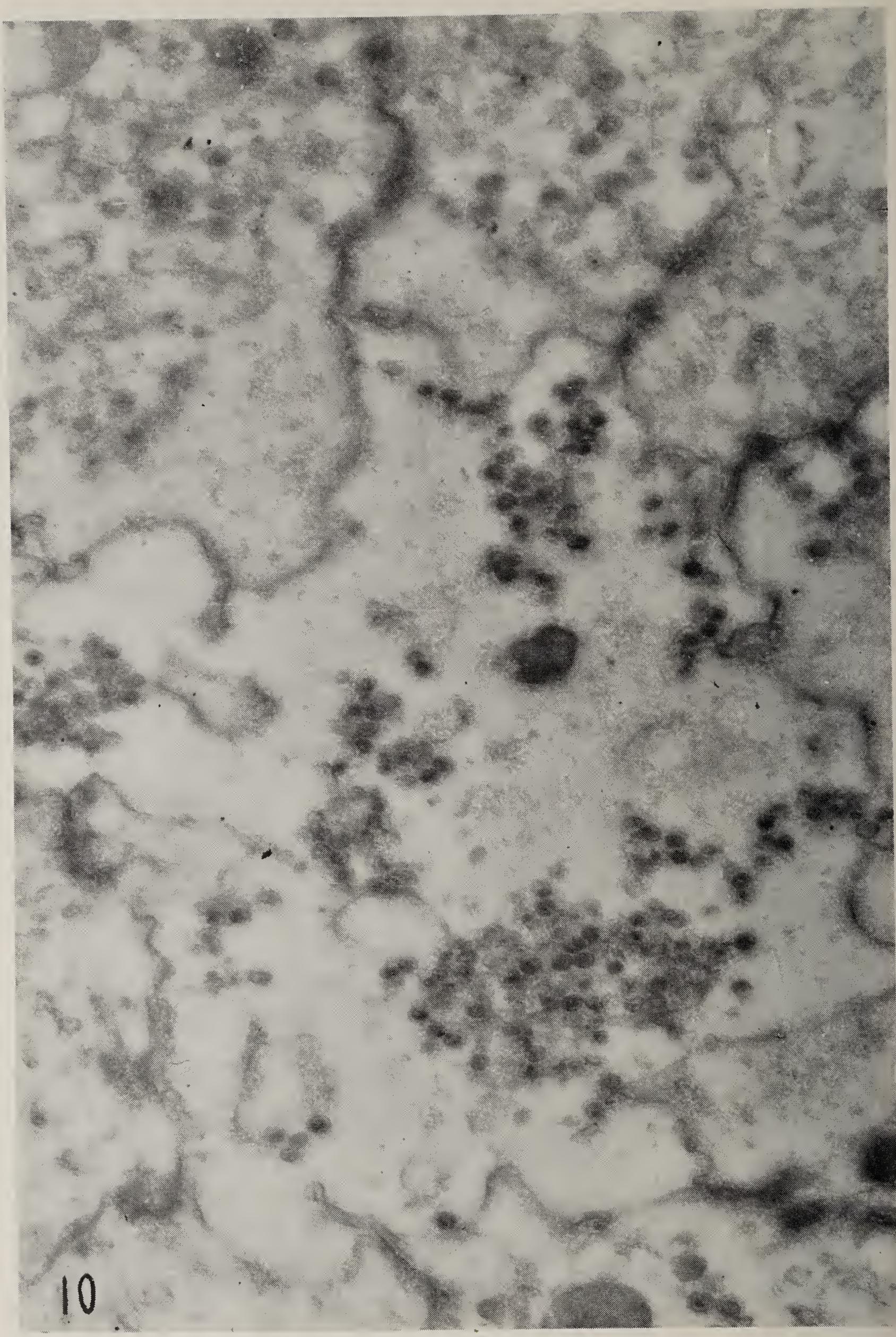


FIGURE 10. Parotid tumor induced in Swiss mouse by extract originating in C3H mammary tumor and then passed in tissue culture. The micrograph shows a lumen containing many virus-like bodies. Larger bodies may be seen within the cells at the top and bottom right. 30,000 X

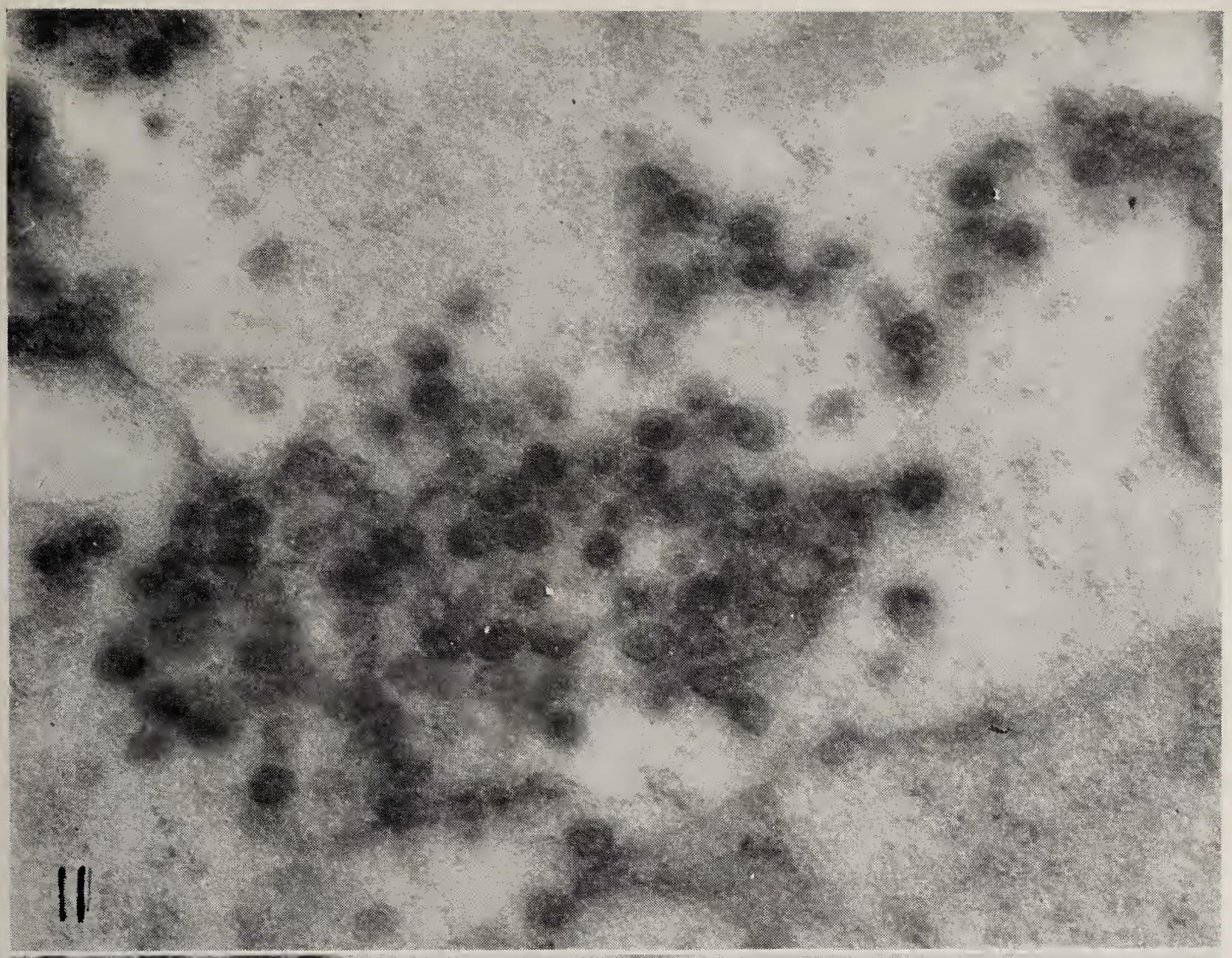


FIGURE 11. A higher magnification picture of a group of particles shown in Fig. 10. A centrally placed dense nucleoid may be seen in some of them. 55,000 X

FIGURE 12. Mammary tumor induced by the same extract. Particles apparently identical with those in Fig. 11 may be seen. 55,000 X

preterminally, and by an elevation in liver fat. No ketosis or ketonuria occurs in association with the hyperlipemia indicating that lipids are utilized to meet an increased caloric requirement.

Other systemic effects shown to occur during the second phase are (1) anemia, (2) enlargements of the liver and spleen, (3) an increase in adrenal size, and (4) liver enzyme changes.

According to Taylor and Pollock ('42) the anemia may occur at a very early stage in tumor growth, or in the case of dye-induced carcinogenesis between the application of the carcinogen and the appearance of the tumor. This effect thus differs from the situation seen clinically where the anemia occurs as a manifestation of advanced cancer.

Enlargements of the spleen and liver are due to increases in protein as well as water content in these organs. In the terminal stage the protein, water and lipid content of liver and spleen return to or decrease below normal levels.

Hypertrophy of the adrenals is associated with involution of the thymus and the retention of salt and water in the tumor-bearing host. Begg ('53) has reported that with growth of the Walker adenocarcinoma approaching 20–25% of the body weight there is a marked decrease in adrenal cholesterol and ascorbic acid suggestive of adrenal hypofunction or exhaustion.

Since the tumor-bearing rat on an *ad libitum* diet shows a decreased food intake and the food that is consumed is partitioned between the tumor and host tissues it would appear that the animal is in a condition of semi-starvation. Dietary studies indicate however that most systemic effects are not secondary to malnutrition nor can they be averted by dietary means.

According to Ingle ('49) the over-all effect of an enforced dietary regime is simply to prolong the first phase of tumor growth. Forced feeding on diets high in fat, protein or carbohydrate may increase the nitrogen in the carcass and may decrease the ratio of tumor to body weight. Even though weight loss is partially offset, other systemic effects persist and may be augmented since the tumors also are larger, e.g. anemia, adrenal hypertrophy and liver enzyme changes. If the tumors reach 25% of the body weight forced feeding is incapable of maintaining nitrogen balance and preventing cachexia.

Metabolic studies on the liver also demonstrate convincingly that the systemic effects of a tumor are not due simply to a dietary inadequacy. Despite an overall decrease in host protein, isotope studies show an increase in the incorporation of P^{32} and formate-C^{14} in liver DNA, and an increased uptake of glycine-2-C^{14} in conjunction with an increase in total hepatic protein. Enzyme changes in the liver are selective rather than general as would be expected if these changes were due to inanition. For example, in starvation there are decreases in liver catalase and xanthine oxidase activities. In the tumor-bearing animal xanthine oxidase activity remains unaltered whereas a profound reduction in catalase activity occurs. Other metabolic changes in the liver include an increase in glycolysis, and a decrease in its capacity to form glycogen (Goranson, 1954, Begg, 1954) which may reflect one or more of the following: an increase in the caloric requirement, a decrease in gluconeogenesis, adrenal hypoplasia and a diminution of hepatic phosphorylase activity.

As pointed out by Greenstein a striking feature of the hepatic changes is that they occur in the direction of a liver undergoing neoplasia. However, the changes are less, quantitatively, and are reversible. The work of Haven and Bloor ('51) indicate that this concept may also extend to changes in the constitution

of fat in the tumor-bearing host. During tumor growth the concentration and composition of each lipid in the carcass tends to approach that of a particular lipid in the tumor. Tumor phospholipid concenttration is high and that of neutral fat is low. Unsaturated fatty acids in the carcass of the tumor-bearing animal are higher than that in a control animal and more like that of the tumor. The ratio of solid to liquid fatty acid in the carcass also resembles that in the tumor. These alterations in the constitution of fat have been shown in experiments in which the diet of controls was adjusted to that of tumor-bearing rats on an *ad libitum* diet.

Mechanisms of Tumor Effects

Attempts to explain the mechanism underlying the systemic effects of tumors have led to two general hypotheses, not, however, mutually exclusive. One theory postulates that the tumor abstracts from the blood some metabolite, or metabolites, necessary for the proper functioning of host tissues. This implies more than the pirating of dietary materials since the tumor grows even in an animal deprived of food at the expense of host tissues. The second theory proposes that the systemic effects are produced by some diffusible substance or substances released by the tumor. The test system most thoroughly studied in advancing this latter hypothesis is hepatic catalase—the liver enzyme which undergoes the most profound change in the tumor-bearing host. Since the physiological role of catalase is not clearly understood the choice of liver catalase has been somewhat pragmatic, the effect, rather than the significance of the effect, being emphasized.

The catalase-depressant effect of tumor is peculiar to the liver enzyme. Renal catalase activity is only slightly lowered and blood catalase (residing in the red cells) is apparently unaffected even when anemia is present. A possible explanation for these differences is given below.

Fractionation studies on liver homogenates (von Euler and Heller, 1949) indicate that catalase is divided almost equally between the mitochondrial and the microsomal-supernatant fractions, the enzyme in the latter fraction being the first to undergo a change in the tumor-bearing host.

The specificity of this tumor effect was shown in the investigations of Greenstein and Andervont ('43) who observed that other factors such as protein-depletion, inanition, and X-radiation, which lower liver catalase exert a similar action on other liver enzymes such as xanthine oxidase. Moreover, liver regeneration, pregnancy, the growth of embryonal implants, even fast growing benign tumors are apparently without influence on hepatic catalase.

The decrease in liver catalase is progressive with growth of the tumor and rapidly returns to normal levels after removal or regression of the tumor. This has been shown by implanting the sarcoma 37 subcutaneously in the tails of mice and subsequently removing the tumors by caudectomy, and also by implanting this tumor under the skin of the I strain mouse in which animal the tumor regresses in approximately 3 weeks.

The experiments cited indicate that the change in liver catalase fulfills the criteria for a systemic effect of a tumor on its host.

The investigation of Lucke provides clear evidence that a circulating factor is involved in the catalase-depressant action of tumors. Adult rats were linked parabiotically and some months after effecting the union one parabiont received an implant of the Walker tumor. While parabiosis alone did not affect the hepatic catalase activity, after implanta-

tion of the tumor a depression in liver catalase occurred to an almost equal extent in both parabionts. The experiments demonstrated the presence of a "toxin" released by the tumor in fairly large amounts, or with a slow rate of disappearance, since it must of necessity pass into the tumor-free animal through relatively small anastomoses linking the circulation of the two animals.

Nakahara and Fukuoka ('48) succeeded in obtaining a chemical fraction from a large number of human tumors which on injection into mice produced a marked diminution in liver catalase, and in addition another characteristic tumor effect, involution of the thymus. The active material, designated "toxohormone", was found to be present in the alcohol-insoluble fraction of tumors and was shown to be heat stable. It was also recovered in the urine of cancer patients and in the gastric juice of patients with gastric carcinoma. Blood sera from cancer patients and from tumor-bearing animals have been found to yield small but detectable amounts of the active substance. The toxic material has also been obtained from normal human and animal tissues but in relatively minute amounts. One of the striking features of the tumor "toxin" is its ability to cross species barriers.

It has been suggested that the relative insusceptibility of renal catalase to the toxin may be due to the rapid excretion of this material. The absence of effect on blood catalase may be attributable in part to the low concentration of "toxohormone" in the circulation.

Tumor fractionation studies were also carried out by Greenfield and Meister ('51). An active, heat-stable substance was obtained in the non-dialyzable, alcohol-insoluble fraction of tumor homogenates and from ultracentrifugal data a molecular weight of at least 40,000 was assigned to it.

On subjecting the alcohol-insoluble fraction to digestion in 6N HCl at 100°C for 12–48 hours Greenfield and Meister also recovered a catalase inhibitor in the dialysate. Digestion of the alcohol-precipitable fraction with papain or pepsin also yielded a dialyzable substance active *in vivo*. According to Nakahara and Fukuoka the "toxohormone" is excreted in the urine in the non-dialyzable form. These investigators suggest that the toxohormone may occur in two forms, one non-dialyzable and the other dialyzable, and that the latter may be derived from the former by an enzymatic hydrolysis.

Further attempts at purification of the active principle were undertaken by Ono and others ('56) who subjected the alcohol precipitate to hydrolysis in 1% pepsin for 4 days. The peptide digest was salted with sodium picrate at one-third saturation and yielded picrate crystals with potent anti-catalase activity *in vivo*. The active picrate gave a blue colour with ninhydrin after one week, contrary to the rapid reaction of free amino acids. It also gave one spot in paper chromatograms, in an acetic-butanol system. As determined by paper chromatography the amino acid composition was reported to be: aspartic acid, glutamic acid, serine, threonine, proline, hydroxyproline, alanine, valine, leucine, isoleucine, phenylalanine, lysine, glycine, and arginine. The molecular weight of this complex was calculated to be of the order of 4000. However, peptides of many species may be assumed to be present in the pepsin digest and there is a strong possibility that some of these could form a mixed picrate crystal with the active component.

As currently conceived the "toxohormone" may consist of a relatively small molecule present in the tumor as a complex, in which form it enters the bloodstream and appears in the urine.

Unlike the "kochsaft factor" of Hargreaves and Deutsch ('52), obtained as a boiled extract of tumor homogenate

and which was reported to bind the iron moiety of crystalline catalase, the "toxohormone" does not inhibit catalase *in vitro*. The mechanism of catalase depression by the cancer agent is believed to be through an interference with the process of synthesis. Since (according to Theorell, 1951) erythrocyte catalase has a much longer life span than the liver enzyme this may also partially explain the relative insensitivity of blood catalase to the "toxohormone" *in vivo*.

Catalase activity is notably deficient in tumor tissue. An interesting suggestion by Nakahara and Fukuoka is that the production of the "toxohormone" by the tumor may be causally related to this enzyme deficiency. This is also in harmony with the concept of convergence of the metabolic pattern of host tissues with that of the tumor.

The studies of Adams ('52) suggest that hepatic catalase is under hormonal influence, and that the tumor-host effect results in an altered response to hormonal factors. Experiments carried out in mice showed that hepatic catalase activity is higher in the male than in the female, and that in animals of both sexes carrying tumors of comparable size, the enzyme is depressed to the same extent. Testosterone was observed to elevate liver catalase in the female mouse and in the castrate male. Liver catalase was depressed in the adrenalectomized mouse but could be restored to the normal level by the injection of cortisone. Cortisone had no effect on the catalase activity in the castrate animal.

The injection of tumor homogenates into normal mice was reported to abolish the effects of both cortisone and testosterone on liver catalase. Since the concentration of "toxin" in a tumor *in situ* is presumably higher than that in host tissues, Adams proposed as a generalization that the tumor factor may render the tumor insensitive to hormonal influence and so contribute to its autonomy.

Conclusion

In the study of tumor-host relationships a focal point of attack has been the alteration of hepatic catalase in the tumor-bearing animal. Whether the "toxohormone" concept that has emerged from studies attempting to establish a chemical basis for this particular phenomenon has a broader significance with respect to pathological changes occurring in the tumor-bearing host presents an intriguing problem.

Questions also arise as to the nature of the metabolic change in the cancer cell leading to the production of the "toxin", and as to the possible effects of the substance on the cancer cell itself.

The progression of systemic changes leading to the wasting syndrome of cachexia can only be averted by removal of the tumor. If the "toxohormone" fraction can be purified and shown to possess antigenic properties it would be of extreme interest to determine if the catalase-depressant and other systemic effects could be counteracted by the injection of specific antibody.

The early hope that some change in the tumor-bearing host might provide a clue to the problem of the early diagnosis of cancer has yet to be realized. In experimental studies employing transplantable tumors the effects investigated appear only when the tumors attain a critical size. A more promising approach may be through the study of spontaneous tumors and the development of techniques for the detection of subtle changes in susceptible sites in the cancerous host.

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AN INTRODUCTION TO THE IMMUNOGENETICS OF TUMOR TRANSPLANTATION

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"Germinal Genetics"—Classical Concepts

THE whole science of genetics rests on the simple fact of sex: a single cell from a male organism, the sperm, fuses with a single cell from a female organism, the egg, and, in so doing, starts a new organism which possesses characteristics derived from both parents. One action of the sperm is trivial, the triggering of division of the relatively enormous egg into small building blocks from which the new organism can be built, trivial because this triggering can often be duplicated artificially by simply pricking the egg with a needle. The other action of the sperm is important—it contributes to the egg an amount of the father's hereditary material approximately equal to that contributed by the mother. Soon after fertilization, the nuclei of sperm and egg fuse and the resulting single egg nucleus thus obtains hereditary material derived from both parents. As the egg divides repeatedly, it gives up a copy of this hereditary material to each of the cells which differentiate to form the tissues and organs of the body. Each time a body cell divides it must also hand down its hereditary material to its newly formed daughter cells.

Genetics deals with the entities that make up the hereditary material—the genes—where they are localized in the chromosomes, how they duplicate themselves, how they are transmitted, what their chemical composition is, how they act to control development, form and function, and they can change (mutate). The basic technique underlying all genetic experiments is the transfer of genetic material from one individual or cell to another followed by the examination of the progeny for the presence or absence of the character(s) derived from one of the parents.

We can recognize two branches of genetics. First, classical genetics which may be referred to as "germinal genetics." This deals only with situations in which the transfer of hereditary material is sexual and is accomplished by mating. And we have already seen that the essential quality of mating, stripped of all its physical and psychical paraphernalia, is the fusion of two germ cell nuclei containing the hereditary material of both parents. But here we are studying how the characteristics of a whole individual are inherited from two other individuals. This is the kind of hereditary phenomenon we are referring to when we remark that baby looks just like his father, or when we speak of a disease running in families. A second, more recent approach to ge-

netics may be called "somatic genetics". It deals with situations in which the transfer of hereditary material is by non-sexual means, as for example, during cell division. Here we attempt to find out about genes in the individual cells of the body. We know that certain characteristics must be inherited from cell to cell, otherwise why would it be that when a cell in an intestinal crypt divides, it gives rise to more intestinal cells, not liver cells or kidney cells? Yet we do not know that the behaviour of genes in body (somatic) cells is the same as in germ cells.

Suppose we wanted to analyze the hereditary characteristic of somatic cells rather than of whole organisms. Our task would be enormously simplified if body cells were endowed with sex, fell in love with each other and settled down to have families. Then we could choose an obvious character present in a "mother" cell (say pigment) but not present in a "father" cell and look for the character in the "baby" cells. If it was there in all the baby cells we could say this is probably a dominant character just as brown eyes are dominant to blue eyes or kinky hair is dominant to straight hair. We could then wait for the boy baby cell to grow up and mate it to its sister cell (there being no taboos against this sort of thing among biologists) and see what happens to their offspring. If three out of every four cells had brown pigment, then we could say that the ability to make pigment is probably inherited in a Mendelian way and is probably due to a single dominant gene.

Let us look at this more closely to see what happened. Every cell would inherit a gene from its father cell and a gene from its mother cell for the character of brown pigmentation. At the beginning of the experiment, let us say that the mother cell, being brown, had two genes for brown, which are dominant—BB. The father cell was colourless and so he had two recessive genes which we will call bb. In the first mating, the mother cell gave up half her genes or B to the baby cell and the father cell gave up half his genes or b to the baby cell. The condition of the parents is, as you know, called homozygous i.e. both genes of a pair are the same (one from the mother cell, the other from father cell) while the condition of the baby cell is heterozygous, that is the two genes of the pair are not the same, one is dominant, the other recessive i.e. Bb.

Now when this baby cell (called the F_1 or first filial generation) later in life gives up half its genes to make a second or F_2 generation baby cell it can give up either a B or a b. And the same for its mate, all happening at random. The F_2 baby cell then can have the following combinations BB, bB, bb and nothing else. You see that any baby cell having B will be brown and any baby cell not having B will be colourless and that the probability of an F_2 baby cell being brown is 3:1. When we see such a ratio of 3:1 in the F_2 generation we can say with some assurance that the inheritance is as Mendel described it nearly 100 years ago, and it is due to the presence of a single dominant gene. If we are not sure yet, we can mate the F_1 cell with one of its parent cells. Suppose we mate an F_1 female cell with her father cell that is colourless. The cross, now called a back-cross, should look like this:

Brown *Bb* x bb colourless

↓

Bb *Bb* bb bb

2 *Brown* : 2 colourless

You see that it comes out 1:1. If in actuality you find that the ratio of brown to colourless cells is 1:1, then you have proof that the inheritance of brown pigmentation in the cell is by a single dominant gene.

Now suppose that you found that instead of an F_2 ratio of 3:1 you got 9:7

brown cells to colourless cells. This would mean that in order for a cell to become pigmented it requires the simultaneous presence of two dominant genes. Here is why:

Brown	*BBCC*	x	bbcc		colourless
		↓			
F_1	*BbCc*	x	*BbCc*		
	Brown	↓	*Brown*		
F_2	*BBCC*	*BBCc*	*BbCC*	*BbCc*	
	BBCc	BBcc	*BbCc*	Bbcc	
	BbCC	*BbCc*	bbCC	bbCc	
	BbCc	Bbcc	bbCc	bbcc	
	9 *BC*	:	7 Bc, bC or bc		
	Brown		colourless		

You have probably noticed if you did not already know it that in the situation where two dominant genes were required, the F_2 ratio was $\frac{9}{16}$ while in the one where one dominant gene only was required, the ratio was $\frac{3}{4}$. Note that $\frac{9}{16} = (\frac{3}{4})^2$.

If three dominant genes had to be present simultaneously in order to confer brown pigmentation on the cells the ratio would be $\frac{27}{64} = (\frac{3}{4})^3$, and in general, the number of dominant genes required can always be calculated from the ratio in the F_2 according to this simple formula:

$$F_2 \text{ ratio} = (\tfrac{3}{4})^n$$

where n is the number of dominant genes that must be present simultaneously in order to permit the existence of a certain character.

Now why should we bother with all this, seeing that somatic cells do not have different sexes in the same body and so probably do not fall in love and settle down to have tissue families? We must obviously go about studying somatic cell inheritance in indirect ways, but the nonsense scheme I have just outlined may help to clarify one of the ways we have for studying these problems.

Cancer Cells as tools for the Study of Cell Genetics

It is rather ironic that some of the most pertinent information about inheritance in somatic cells of mammals has come from studies on cells that are known to have abnormal hereditary equipment—cancer cells.

Cancer cells have one reliable and useful character—they continue to grow in an animal unless something drastic stops them. This feature of progressive growth and the ability of an animal to stop this growth has been put to work to tell us much of what we know about inheritance in mammalian somatic cells. How this came about makes a rather interesting story.

In 1889 Hanau first transplanted a tumor. It was a metastasis from a spontaneous cancer of the vulva of a white rat and he transplanted it into the scrotum of another rat. It grew progressively. Five years later Morau successfully transplanted mouse mammary tumors to other mice. But the results were by no means uniform. Sometimes the tumors would grow progressively and kill their hosts; sometimes they would grow for a while and then stop and regress. These unpredictable results gave rise to many theories that could not be substantiated.

In 1902 a break came. In the laboratory of Leo Loeb there existed some mice belonging to the Japanese Waltzing strain. These mice, of oriental ancestry, had a peculiar dance-like gait which was due to a defect in their cochleae. This particular behaviour had been maintained by mouse fanciers through inbreeding—a trick that geneticists use to perpetuate a hereditary trait.

One of these Japanese waltzing mice developed a mammary tumor and Loeb transplanted it to a number of other Japanese waltzing mice. The tumors grew progressively and killed all the hosts but

failed to grow in ordinary white laboratory mice. These findings were verified by Tyzzer in 1909. It soon became apparent from the work of Little and Tyzzer that the uniformly successful results of tumor transplantation in Japanese waltzing mice were due to the fact that this was an inbred strain of mice.

The Meaning of Inbreeding

Mice and men are normally heterogeneous with respect to many of their hereditary characters. This means that individuals differ from one another in the kinds of genes they carry on their chromosomes and also, within a given individual, the two members of each pair of chromosomes may be different from each other, that is, the genes derived from the mother may be different from the ones derived from the father (heterozygous). If one wants to make a population of mice more homogeneous genetically, one can do this by mating a brother with a sister and when their offspring grow up, brother and sister are mated again and so on. At mating, each of the two parents gives up half of its chromosomes to each of the offspring. Thus the offspring, having genes from both parents are more similar to each other than the parents were. Mating a brother with a sister will again produce offspring that are more similar to each other than were their parents to each other, and much more similar than were their grandparents to each other. Similar genes are thus concentrated, different ones are eliminated. This process is also facilitated by the exchange of parts of chromosomes derived from the mother with parts derived from the father that occurs at each generation when the organism is preparing to make sperm and ova.

After some 12 such generations of brother and sister mating, one can consider the heredity of the mice fairly homogeneous, the degree of homogeneity being increased with increasing inbreeding. After about 30 generations the strain consists of mice that are about as closely related to each other as identical twins are. You have heard of successful skin grafts and even of kidney grafts between identical twins—this is the same idea with tissues transplanted from one member to another of the same inbred strain. These mice are not only like each other but the genes on their two sets of chromosomes are identical to each other, i.e., they are homozygous.

There are now a large number of inbred strains of mice available. Some of the best known ones are the A, the C3H, the C57BL and the DBA. Each has its own inherited peculiarities. They have all been inbred for 60 to 100 generations. The equivalent pedigree in man would date back to biblical times.

The Genetics of Tumor Transplantation

Let us examine the kinds of results that can be expected when one transplants tumors arising in a mouse belonging to an inbred strain.

1. Transplants from one member of an inbred strain to other members of the same strain (isotransplants) grow progressively and kill all hosts.
2. Transplants from a member of an inbred strain to a member of an unrelated strain whether the latter is inbred or not (homotransplants) do not grow or grow temporarily and then regress.
3. Transplants into F_1 hybrid animals, where one parent was inbred and susceptible, grow progressively and kill all hosts.
4. Transplants into F_2 mice and into backcross mice from the mating F_1 x resistant parent, grow in a fraction of the hosts only. The susceptible percentage may vary within wide limits according to the strains of mice and the tumor employed.

5. A tumor arising in an F_1 hybrid will grow in all F_1 hybrids but not in members of either parental strain. It will grow in a fraction of F_2 and backcross mice.

If the tumor is transplanted to both kinds of backcross animals produced by mating an F_1 animal with an animal from either of the parent strains, one can, from the ratios obtained, get an estimate of how many of the dominant genes active in the tumor were derived from each of the parent strains.

Thus, in order to learn something about the hereditary characteristics of tumor cells, instead of mating "male" cells with "female" cells as in our nonsense scheme, we have mated a male animal from one strain and a female animal from another and watched the outcome of transplantation of tumor cells into such first and second generation hybrids and into backcrosses of F_1 hybrids mated to both parental strains. The F_1 hybrid animals are all alike and possess all the genes from both strains and we found that such hosts were compatible with cells from both parental strains. The F_2 hybrid animals must differ from one another. For any given gene, about a quarter of them have both alleles derived from the maternal strain, about a quarter have both from the paternal strain and about a half have one allele from each parental strain. If we transplant a tumor from one of the parental strains into a number of these F_2 hybrids and find that 3 out of every 4 animals succumb to the tumor and in 1 out of 4 the tumor regresses, it is fair to conclude that in that particular strain combination and for that particular tumor, a difference of only one gene between the strain of origin of the tumor and the other parental strain decides the outcome of transplantation of the tumor into the F_2 hybrids. If 9/16 $(=(\frac{3}{4})^2)F_2$ hosts support the growth of the tumor, then the simultaneous presence of two dominant genes in the host determine whether or not the tumor will grow; if 27/64 $(=(\frac{3}{4})^3)$, then 3 dominant genes are involved, and so on. The inheritance of susceptibility or resistance to a transplanted tumor is thus Mendelian and depends on the simultaneous presence of dominant genes.

This idea was first suggested by Little in 1914. It was called the "genetic theory of transplantation". The theory was extended by Little and Strong in 1924 and has since been consolidated and confirmed by many investigators. It applies not only to the transplantation of mammary adenocarcinomata but has also been shown to apply to ovarian tumors, leukemias, sarcomas and melanomas. The genes postulated in this theory have been called "histocompatibility genes" (H-genes). The prefix "histo" is used because there is reason to suppose that the same genes determine susceptibility and resistance to the transplantation of both neoplastic and normal tissues.[1]

If the host has the same H-genes as the transplanted tissue, it will accept the transplant because it is confronted only with genes (or their products) that are already part of itself. If the transplant is of normal tissue, it will persist indefinitely; if it is of tumor tissue, it will grow progressively and kill its host.

It does not matter whether the host has any other H-genes that are not present in the transplanted tissue, as long as the H-genes in the transplant are also present in the host. For this reason it is possible to transplant tissues successfully from two different inbred strains into a host which is an F_1 hybrid between these strains. However, should the cells of the transplant contain any genes that the host does not also have (and therefore cannot recognize as its own), the host will react in such a way as to reject the transplant. Thus the tissues of an F_1 hybrid between two strains cannot be successfully transplanted into either of the parent strains because the hybrid tissue will contain H-genes from the op-

posite parent strain that are not present in the parent strain host that is being tested.

The genetic laws of transplantation apply equally well to the transplantation of normal tissues as to tumors. This makes one wonder why it is impossible to transplant skin successfully from a human mother (or father) to their children. The reason for this is that human beings are not inbred, therefore the H-genes on the two members of any pair of chromosomes are not necessarily the same. Since only half of the mother's chromosomes are given to the child via the egg, the child will not possess many of the H-genes that are present in the mother's skin cells. As mentioned earlier, whenever such a situation arises, the transplant is rejected by its host.

Generally, normal tissue transplants appear to demand the presence of more matching histocompatibility genes in order to survive in their hosts than do tumor transplants. Snell has suggested that 14 is a conservative estimate for the total number of H-genes in the normal mouse. That is, in order for a normal tissue transplant to "take", at least 14 H-genes must match in donor and host. There are probably more. It is easy to see why the conditions for compatibility in tissue transplantation are much more difficult to satisfy than are those of blood transfusion where only a few groups have to match. It is obviously nearly impossible to find two non-inbred individuals, even closely related ones, in whom 14 or more H-genes are identical.

Fine Structure of Histocompatibility Loci

Three of the histocompatibility genes in the mouse have been subjected to detailed study. To do this it was first necessary to "isolate" these genes from the rest of the genotype. Snell accomplished this when he developed by controlled breeding a number of sublines from an inbred mouse strain in which all the genes were the same as those of the inbred strain except one, and that one was a histocompatibility gene. For example, one such subline was identical with the A strain in all its genes except one. At the H-2 locus on the 9th chromosome it had a gene derived from the Swiss instead of from the A strain. Therefore a tumor from the A strain which required the presence of only one matching H-gene in the host to ensure progressive growth, would not grow in the subline identical to the A strain except for the one H-gene introduced from the Swiss. These sublines are called "isogenic resistant lines" or "IR lines" and there are now 26 of them that have been carefully analyzed.[2] Most of them differ from their inbred strain of origin only at the H-2 locus (linked to the T and Fu tail anomaly genes on the 9th chromosome); others differ only at H-1 (linked to a coat color gene C on chromosome 1) and others differ at H-3 (linked to another coat color gene A on chromosome 5). These animals are homozygous, and therefore in an IR line both members of a pair of chromosomes will bear the same H-gene. And these will be present in all the cells of the body. Thus tissues from a mouse of an IR line will be freely transplantable to other mice of the same IR line. If, however, a mouse of an IR line is mated to a mouse of the strain of origin of the IR line, the offspring will be heterozygous for that H-gene and these F_1 tissues will not be transplantable to either of the parental strains. Here the incompatibility is due to only one gene, all the others being identical with those of the parental host. In spite of this, Klein[3] has shown that tumors arising in such F_1 animals are totally destroyed by the parental host, while cells containing the compatible H-gene survive. By mixing small numbers of compatible cells with massive numbers of cells incompatible because only one H-gene is

different, he showed that the system was able to detect specifically the presence of a single compatible gene on a single chromosome in a few compatible cells out of a total population of many millions of incompatible cells. All of the latter were destroyed by the host and the few compatible cells were able to grow progressively and form a massive tumor.

Tumor Histocompatibility Genes

The number of H-genes required in the host in order to permit growth of different tumors varies from 1 to 13. Two tumors arising spontaneously in the same animal may have different histocompatibility gene requirements, and therefore probably different H-genes are active in their cells. The value obtained for the number of H-genes required for a given tumor is not necessarily constant at different periods in its history. Strong in 1926 showed that a certain tumor that arose in the DBA strain, when first tested, required the presence of 7 matching H-genes in the host for progressive growth to occur. With repeated transplantation in the DBA strain, the H-gene requirement of the tumor dropped to 2 genes, then to 1 gene. Decreases in the H-gene numbers (actually detected as stepwise increases in the proportion of F_2 or backcross mice capable of supporting the growth of the tumor) regularly occur during the transplantation of tumors. No increases in the number of matching H-genes required for growth have ever been observed when tumors have been grown in their strain of origin.

There is some indirect evidence that the active H-genes may not be the same in all the cells of the same tumor.

The H-genes are not all equally important; some are stronger than others in the sense that they play a more significant role in determining whether virulent tumors will grow when the H-genes of the tumors do not match the H-genes of their hosts. Thus some tumors that have been transplanted for many generations will grow progressively in a host in which only one H-gene matches that of the tumor but if this does not match the tumor will regress. The strongest of these H-genes is the H-2, which is known to be situated in the 9th chromosome of the mouse, at a small distance from two known genes that control tail structure. This close linkage is deduced from the fact that it is possible, by suitable mating, to introduce into the genotype of a tumor-susceptible, normal-tailed mouse, an H-gene which is different and therefore confers resistance to the transplantation of a tumor in its host and, simultaneously, a gene causing a tail anomaly in the mouse.

Applications of Transplantation Genetics

The genetic laws of transplantation have had a rather wide variety of applications. Most useful has been the fact that it is possible to transplant tissues from either or both parental strains into F_1 hybrid animals, but not vice versa.

In this way it has been possible to answer the question of whether the high susceptibility of certain strains to the induction of certain tumors resides in the tissue where the tumor arises or in the host generally.[4] To do this normal tissue from a high and from a low susceptibility strain had to be simultaneously transplanted to a single host, thus ensuring an identical environment for the two tissues. Normal mammary glands, ovaries or lungs, from strains with high and low susceptibility to tumor induction were thus transplanted to hybrids between these strains. The hybrids were then subjected to the carcinogenic stimulus. In each case the incidence of tumor was found to be high in the transplant from the highly susceptible parent and low in the transplanted tissue from the parent with low susceptibility. Since the host

environment in which the carcinogenic agent acted was kept constant in all these experiments, it is fair to conclude that the inherited susceptibility to tumor induction resides in the tissue that itself becomes malignant.

Klein[5] was able to show that the induction of lymphosarcoma allegedly due to chromatin fragments was actually due to contamination of the chromatin preparations by whole tumor cells. He did this by using chromatin material from a parental strain injected into an F_1 hybrid. The resulting lymphosarcoma grew in both the hybrids and the parental strains. Had it been a true induction in the hybrid, the growth of the tumor cells would have been restricted to F_1 hybrids.

Kaplan[6] showed that X-irradiation can cause leukemia by acting indirectly on the host rather than directly on the normal cells that later become leukemic. This was done by irradiating F_1 hybrids and transplanting normal thymus from parental strains to these animals. Leukemia developed in the normal thymic transplants as proved by the transplantability of the leukemic cell into both F_1 hybrids and parental strains.

These and a large number of other experiments have utilized the information embodied in the genetic laws of transplantation.

Immunogenetics

We have seen that the H-genes all behave like dominant genes, that is two different H-gene alleles, when present in the same cells on the two members of a single chromosome pair (heterozygote), both express themselves. This is seen, for example, in the case of a tissue from an F_1 hybrid transplanted into a parental strain. The two H-genes at a given position on the chromosome pair are different because they are derived from different strains, yet one of the two is identical with the H-gene of one of the parent hosts. If one H-gene were dominant and one recessive, it should be possible to transplant the hybrid tissue into the one parent strain carrying the dominant allele but not into the other carrying the recessive. We know that it is impossible to transplant the hybrid tissue successfully into either parent, therefore both H-genes of a homologous chromosome pair must be expressing themselves.

This is unlike the situation encountered in classical Mendelian inheritance where only the dominant allele in a heterozygote expresses itself. It is, however, precisely the condition found in the inheritance of blood groups. Thus in a heterozygous individual carrying the allelic genes that determine the presence of both antigens A and B on the red cells, both antigens A and B are actually found —the red cells are agglutinated by anti-A as well as anti-B serum.

This similarity in genetic behaviour between tissue transplants and red blood cells is not fortuitous. Gorer[7] has shown that the same gene (H-2) that determines the outcome of transplantation of a strain A mouse tumor into F_2 hybrid mice also determines a blood group (Antigen II) on the red cells of normal mice of the A strain. He accomplished this by simultaneous genetic, transplantation and serological studies. He used the presence of a tail anomaly as a marker for a specific region of a chromosome that also carries the H-gene determining the success of transplanting a tumor from the A strain into F_2 hybrids. Then he tested the red cells of the F_2 hybrids with antisera prepared against strain A tissue. Those hybrid individuals whose red cells had the antigen (Antigen II) also supported the growth of the tumor, and did not have the tail anomaly while those whose red blood cells were not agglutinated by the anti-A strain antiserum rejected the A-strain tumor and had the tail anomaly.

Because of the close relation between the genetic conditions of the graft and the immune response of the host, a new name has arisen for this area of research. It is "Immunogenetics."

Tumor cells from a given strain can stimulate, in a foreign strain, the production of antibodies directed against the antigens of red cells from the strain of origin of the tumor. Tumor cells can also absorb antibodies out of a serum produced against the normal tissues of the strain of origin of the tumor. Therefore tumor cells have the same antigens as the normal tissues of the same strain (and they may have others that differ from those of normal tissue) though they may not have all of them. By systematic studies using these techniques of red cell agglutination and antibody absorption along with transplantation into genetically controlled animals in a wide series of strain combinations, it has become possible to "type" the tissues of mice of a large number of strains. At least 12 antigens are now known in mice and different combinations of these are characteristic of different groups of mouse strains. Thus the AKR and the C3H strains have antigens C, E, H, and K, while C57BL and DBA/2 have C, D, E, F, H and M. The interesting feature of all this is that all of these antigens in any one strain are determined by one locus, the H-2, on the 9th chromosome; different strains carry different alternative forms of the H-2, thus have different combinations of antigens. Since each antigen is probably determined by a single gene, the H-2 locus is probably a region of the 9th chromosome consisting of a rather large number of closely linked genes which are usually inherited as a single lump. The same complexity will probably be found for other histocompatibility genes situated on other chromosomes and therefore inherited independently of the H-2.

Tumor Chromosomes

Recent advances in technique have permitted preparations to be made of the chromosomes of normal and tumor tissue so that they can be clearly distinguished. So far no malignant tumors have been found that have perfectly normal chromosomes. The abnormalities may be in number and/or structure. All cells in a normal tissue have the same number of chromosomes or simple multiples of that number, e.g., the germ cells of the mouse have exactly 20, most of the body tissues have exactly 40 and some tissues, like liver, have 40, 80 or 160 chromosomes per cell. Mouse tumors may have 40 chromosomes in most of their cells but many cells of the same tumor will also have 41, 42 or more. Some tumors may have no cells with 40 chromosomes but most or all cells will have fewer or more than 40. Others have around 80 chromosomes in most of their cells, with the rest of the cells grouped around 80 or some number near 80. Abnormalities are also frequently observed in the chromosomes of tumors—they may become broken, and remain broken, or they may become fused with parts of other broken chromosomes, or they may stain in an unusual way.

Each tumor seems to have its own numerical and structural chromosomal individuality and variations in the chromosome number from cell to cell within any given tumor appear to be the rule. Hauschka and Levan[8] have made the generalization that tumor cells in which drastic changes in the chromosome number have occurred are usually able to grow vigorously in a foreign strain, that is, they do not respect the genetic laws of transplantation. It has also been found, in keeping with this concept, that in such tumor cells many of the normal antigens have become weakened or have disappeared.[9] In contrast, the growth of tumors with nearly normal chromosome

numbers are usually sharply restricted to the strain of origin of the tumor and the hybrids expected from the genetic laws of transplantation.

The Homograft Reaction and Tumor Immunity

What happens when the H-genes of the tumor do not match the H-genes of the host into which the tumor is transplanted? The evidence available today points to the idea that under these circumstances the host reacts to the foreign tumor with an immune response. If the genes are identical, the host does not recognize the tumor as being foreign and no immune reaction results. The tumor continues to grow and kills its host.

If a tumor of one strain is injected into a host of another strain it may not grow at all. In this case the immune response develops so rapidly that the tumor cells are killed before they have a chance to get started. Often the tumor will grow up to a point, stop, and regress. Here the immunity has lagged somewhat but eventually caught up and killed the tumor. Sometimes the tumor, although foreign to the host, grows so rapidly that the host does not have time to mobilize its defences before it is dead. Long transplanted tumors with irregular high chromosome numbers often behave in this way. In the last two cases, if the host is challenged with the same tumor a second time it is found to be immune and little or no growth of the tumor occurs. The sequence of events that results in the rejection of an incompatible graft of either normal or tumor tissue is called the homograft reaction.[10] As we have seen it is due to antigenic differences (determined by differences in histocompatibility genes) between graft and host and a difference at a single locus on a chromosome is sufficient to bring about a violent reaction by the host with destruction of the graft.

The destructive process may take at least 3 forms, depending on the nature of the graft. Solid tumors and skin grafts become vascularized by the host, then, by one week, small mononuclear cells infiltrate the graft, circulation stops, and the graft undergoes infarction. Ascites tumors do not become vascularized but proliferation of cells occurs at the margin of the graft leading to the production of a large number of macrophages which form a syncitium and encircle individual tumor cells. These are destroyed and the débris is phagocytosed. Leukemic cells undergo lysis before any host leukocytes invade the graft. The speed of the destructive process is greatly enhanced if the host has been previously exposed to foreign antigens of either the graft itself or other tissues from the strain of origin of the graft. This acceleration is specific and occurs only when the same antigens are used for the preimmunization as the antigens in the cells of the graft.

Although it is clear that the homograft reaction is essentially immunological, it has not always been possible to detect circulating antibodies to grafts that are undergoing destruction by the host and passive transfer of transplantation immunity has succeeded only under special conditions.

Probably the successful homograft reaction requires the operation of complex processes involving one or more of the following:

1. Production of one or more kinds of antibody by host plasma cells under the direct or indirect influence of the antigens of the grafted cells. 2. A tuberculin-type of allergy in which a circulating soluble antibody very quickly becomes fixed to cells after which it is no longer detectable in serum. Combination of this antibody with antigen at the cell surface would then produce the destructive allergic response. 3. Direct killing and lysis of incompatible cells by freely circulating antibody and complement. The mechan-

ism seems to be the same as that of the lysis of red cells after incompatible blood transfusion. 4. Persistent inflammatory reaction permitting transport of antibody by small mononuclear cells into the incompatible graft. 5. Combination of antibody with graft cells facilitating phagocytosis by host macrophages. This would be similar to the coating of incompatible red blood cells by Rh antibody resulting in their phagocytosis by host macrophages.

There is thus a considerable though by no means complete body of fact and theory regarding the reaction of a host to a tumor (or normal tissue) graft derived from a donor with a different genetic constitution.

Usually, as stated before, tumors transplanted into animals of the same strain grow progressively in all their hosts. But the reaction of the hosts to different kinds of tumors seems to be different. Tumors induced with a chemical carcinogen can act as antigens and evoke a state of immunity in their isologous hosts, so that a second injection of the same tumor is rejected.[11] This immune reaction does not develop against cells of a spontaneous tumor. This must mean that during induction of the tumor with a chemical carcinogen, new antigens have appeared in the cells. These new antigens are known to be confined to the tumor cells; they are not detectable in the nontumorous tissues of the same animal who was treated with the carcinogen. These new antigens (or the mutated genes responsible for them) must be handed down by the tumor cells to their daughter cells at mitosis, for after transplantation, these tumors are still capable of immunizing their isologous hosts.

Not only can new antigens appear during induction of tumors with chemical carcinogens, but they may also develop during growth of these tumors. Some of these antigenic changes have been shown to be due to mutation of single histocompatibility genes during transplantation of the tumors.[12]

When we turn to the question of whether any immunity exists to the cells of a spontaneous tumor in its original host (autologous), we are in almost total ignorance. Clinical observations of spontaneous regression of cancer in man have sometimes been interpreted as indicating that such an immunity does exist but these observations for the most part can be accounted for in other ways.[13]

One approach to the problem of whether the cells of a spontaneous tumor are able to induce an immune reaction in the host in which the tumor developed would be to try to find out how many tumor cells are necessary in such a host to start a successful transplant. This number should be somehow related to the degree of immunity, if any immunity exists.

Quantitative Transplantation

If all tumor cells survived the manipulations necessary for preparing suspensions and transplantation and were identical to one another in their ability to give rise to progressive growth there would be no necessity to specify the size of any tumor cell inoculum. The same laws would then apply to the transplantation of a single cell as to 10^8 cells. But tumor cells do not all survive dissociation and transplantation equally well and there is good evidence that they are not all equally capable of giving rise to progressive growths, even when they belong to the same tumor.

A number of dyes are known that are able to penetrate cells only if the cells are dead. Eosin, erythrosin, trypan blue and nigrosin are examples of such dyes. If a suspension of cells is prepared from a tumor and mixed with a low concentration of one of these dyes, some cells will rapidly become coloured and the rest will remain colourless. By counting the colourless cells in a hemocytometer,

one can obtain a measure of the maximum number of viable cells in the known volume of suspension—maximum because it is impossible by present techniques to know whether all unstained cells are equally viable and whether they are all equally capable of multiplication. In some tumors the proportion of unstained cells is very near 100%, in others, it may be as low as less than 10%.

Some transplantable tumors do not have to be dissociated artificially because they naturally exist as free cells suspended in fluid. These are called "ascites tumors."[14] Their cells grow and divide in the ascites fluid of the peritoneal cavity from which they can easily be removed, counted and transplanted to the peritoneal cavities of other animals. Ingenious techniques have been worked out, similar to those used for measuring plasma volume by dye-dilution, for determining the total volume of ascites fluid in the peritoneal cavity. Reliable tumor cell growth curves have been made in this way and have thus paved the way for a large variety of quantitative studies on tumor cells including their biochemistry and metabolism, and the effect of chemical and physical agents on their growth.

A single cell of an ascites tumor can be isolated from a suspension under direct microscopic vision and successfully transplanted to another animal in whom it can give rise to a massive ascites tumor and death in less than 1 month. Most tumors require more than one viable cell in order to start a progressive growth. Even in the case of the most virulent ascites tumors, the experiment described above with a single cell is successful only about 15% of the time.

A simple method of determining one aspect of a tumor's virulence is to make graded dilutions of counted cell suspensions from the tumor, inject these into groups of animals that are genetically identical, and count the numbers of animals that develop tumors. The minimum cell dose necessary to establish a progressive growth is found to vary widely from tumor to tumor—from one cell up to tens of thousands of cells may be necessary for different tumors. Generally speaking, tumors that have been transplanted many times require fewer cells for successful transplantation than do tumors that have had short transplantation histories.

On the other hand, the same tumor in different environments may behave differently with regard to the number of cells necessary for successful transplantation. It is this fact that can be exploited in the detection of host immunity. Solid spontaneous mammary tumors have been surgically removed from mice, cell suspensions have been made, and known numbers of unstained cells have been injected back into their original hosts and into other genetically identical animals of the same age, but not bearing a spontaneous tumor (isologous hosts). Preliminary results of such experiments indicate that the animal from which the tumor had been removed (autologous host) provides a more favourable environment for the growth of its tumor cells than do isologous hosts.

This is a rather surprising result. One might have expected that the growing spontaneous tumor would have acted as an antigen and that the animal bearing the tumor would have developed antibodies against it. When a small dose of the animal's own tumor cells were then grafted back to the same animal, one would have expected these cells to have been destroyed if the animal were able to produce an effective immune response. Instead, the cells grew better than in animals that had not previously been exposed to the tumor cells. Perhaps the classical kind of immune reaction does not occur in an animal when a tumor starts spontaneously in it; or perhaps the immune mechanism of the animal becomes impaired when a spontaneous

tumor is developing. Further application of the technique of quantitative transplantation of spontaneous tumors into genetically controlled animals should yield important information about tumor immunity. It may also help us find out why tumor cells metastasize, for, after all, metastases are really self-imposed autologous transplants of relatively small numbers of viable tumor cells.

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IMMUNOCHEMISTRY

B. CINADER

Immunology has its historic roots and its development within the study of infectious diseases and the study of the synthesis of molecules in response to foreign substances on or released by the invading organism. The body reacts to the presence of these foreign molecules or cells (such as bacteria) by the formation of an antibody, which can neutralize the biological action of the invading agent. The inducer of the antibody response, called an antigen, can be any foreign macromolecule of molecular weight greater than 10,000. The properties of antibodies result from the adaptation of the antibody molecule to the configuration of the antigen and include:

(1) ability to combine *in vitro* with the antigen against which they have been formed.

(2) ability to inhibit the biological action

of antigens such as toxins or enzymes (1). (3) combination with the molecule against which it has been formed and with other molecules containing one or several chemical groups similar to those of the inducer. Antibody will distinguish its inducer from other antigens which may differ by as little as one atom, corresponding to a difference of only 1 Angstrom. We refer to this property as the "specificity" of the antibody.

The antibody response itself is as interesting as the substance which arises from it. Its most remarkable features include:

(1) The quantity of antibody synthesized in response to the same stimulus varies from one animal to another and seems to be genetically controlled.

(2) As a rule, the antibody response cannot be elicited by isologous circulating macromolecules.

(3) The antibody response seems to have a "memory"; it is slow and leads to the production of very little antibody when the antigen is injected for the first time. When the antigen is injected for the second time, several months later, the antibody is produced much sooner after this second injection and the quantity of antibody synthesized is much greater.

(4) Antigen which would elicit an antibody response in an adult animal, will fail to do so in an adult which has been injected with this antigen shortly before or shortly after birth. We refer to this phenomenon as acquired immunological tolerance.

Functions of antibodies, other than the protective ones have become evident in recent observations; they can act, themselves, under certain circumstances, as the causative agent of disease, (haemolytic disease of the newborn, allergy, Hashimoto's disease). In all of these manifestations certain tissues of the body react to the presence of new substances which are either derived from external sources or released by other tissues within the body. In view of the many-sidedness of antibody function, the nature of antibodies deserves study as a biological phenomenon of the adaptation of an organism to a change in its internal environment.

Immunochemical techniques can be used in a variety of biochemical and biological fields. It is, for instance, possible to study the homogeneity of any macromolecule (protein or polysaccharide) in terms of the antibodies formed to it. It is similarly possible to follow any changes in a macromolecule in synthesis, activation or evolutionary development—by means of the clues provided by the antibodies reacting not only with the molecules which have induced their formation but also with molecules sharing some structural features with the inducer.

It is difficult to do more than sketch here a few of the immunochemical problems very briefly. I shall try to give some idea of the modern techniques available for the analysis and characterization of antibodies and antigens and of some currents of research directed towards the uncharted depth of the specificity and inheritance of the antibody response.

The Reaction of the Body to a Foreign Macromolecule

The first time an antigen, for instance human serum albumin, is introduced into the blood stream of a rabbit, its concentration decreases rapidly during the first forty-eight hours. From then on its amount falls at a slow but steady rate for nine to twelve days. At the end of this time a sudden dramatic increase occurs in the rate at which the macromolecule is eliminated and after one or two days no further antigen can be demonstrated in the bloodstream. When no antigen is left in the circulation the appearance of a new substance can be demonstrated. Its existance can be observed if the serum is allowed to react with the anti-

gen *in vitro*. The mixture becomes opaque and a heavy flocculent precipitate separates. If the antigen is part of a large cell, say of an erythrocyte, then a suspension of erythrocytes will be clumped (agglutinated), or, in certain circumstances, the erythrocytes will be lysed by the serum. If the antigen is an enzyme, its activity will usually be reduced (1).

The active substance which is found in the serum and which has been synthesized in response to the macromolecule, is called an *antibody* and substances which provoke the synthesis of the antibody are referred to as *antigens*. Most macromolecules, irrespective of their chemical structure, can act as antigens. Proteins, polysaccharides, lipoproteins or lipopolysaccharides, and even some synthetic macromolecules such as polyvinyl pyrrolidone can act as antigens. There thus appears to be virtually no chemical limitation as to the structure or composition of a macromolecule which can elicit an antibody response. While there is no known chemical limitation to the composition of an antigen, there are two other very interesting limitations.

The first concerns the size of the macromolecule. A substance must have a large molecular weight of at least 10,000, in order to act as an antigen. This however, does not mean that the antibody cannot react with a molecule of smaller weight. In fact, the area of the antigen-molecule to which the antibody is adapted appears to be very much smaller. In polysaccharides, it is quite certain that the antibody does, only combine with an area corresponding to one to five units of sugar. However, these individual sequences of sugars must be contained within a molecule consisting of many hundreds of individual sugar molecules before the antibody adapted to the one to five of these units can be formed.

Thus the antibody can react with a small unit, which we call the *hapten*, but antibody synthesis can be induced only by this hapten being contained in a very much larger unit which we call the antigen.

The second limitation to antigenicity is of a much more subtle nature. We have already said that antibodies can be formed to almost any natural chemical structure and apparently even to completely synthetic molecules of a structure quite unlike anything that occurs in the normal body. However, a molecule in order to be an antigen, must be derived as a general rule from a source foreign to the organism that produces the antibody to it. This limitation has been discovered by Ehrlich and has been described by him as "horror autotoxicus". Though this principle of exclusion, of this fear of self-intoxication by the synthesis of antibodies to one's own tissue, remains essentially valid, a number of very important exceptions to the rule have been discovered and will be mentioned later on.

We have, so far, seen what molecules can stimulate an antibody response and how this response affects the fate of the antigen in the circulation. We shall now examine the precipitation *in vitro* between antigen and antibody as one example of the interaction between these reactants and as one method for the assay and characterization of antigens and of antibodies.

The Reaction *in vitro* between Antigen and Antibody

Antigen and antibody can precipitate with one another. The quantity of this precipitate can be assayed. This has become the basis of a method by which antibody can be measured in terms of its nitrogen-content and by which the combining properties of antigen and of antibody can be studied (2). Furthermore, by allowing the reaction to occur

in gels, the components of complex antigen-antibody systems can be observed individually and can be enumerated.

If different, increasing quantities of an antigen are mixed with constant quantities of antibody in a series of tubes and if these mixtures are placed at 37°C and then observed, it is found that after an interval of several minutes or sometimes several hours, one solution becomes opaque. Soon this opacity is also observed in neighbouring tubes and after a further few minutes fine floccules can be seen which become larger and finally precipitate. One mixture precipitates before all others. The mixtures on the left or right of this mixture (containing respectively a little less or a little more antigen) precipitate subsequently and other neighbouring solutions follow suit.

The reaction which I have just described, the precipitin reaction, underlies most other quantitative tests for antibodies.

We refer to the solution which has first precipitated as containing antigen and antibody in *optimal proportions*. We can, by assigning some arbitrary value to the antigen solution, deduce a relative value for the antibody solution. We can deduce relative potency values for antigen solutions of unknown concentration by determining the optimal mixture of the unknown antigen solution with the standard solution of antibody.

In this method the first precipitating mixture and its flocculation time are observed; results are relative and semi-qualitative. The precipitation of antigen with antibody can yield precise quantitative results if the reaction is allowed to go to completion, the precipitates are separated, and the total amounts of precipitated nitrogen are measured. The resulting values can be plotted as a function of the antigen-nitrogen initially added to each solution. The curve so obtained goes through a maximum of nitrogen precipitated corresponding to an amount of antigen-nitrogen added. From this curve the total amount of antibody-nitrogen contained in unit quantity of a serum can be determined. Further information can be obtained by examining the supernatants which are left behind after the precipitate has been removed and assayed for nitrogen. Supernatants may contain either antigen or antibody and this can be determined by adding antibody or antigen to them. Some of the supernatants precipitate with antigen but not with antibody; other supernatants precipitate with antibody but not with antigens and a very few supernatants precipitate neither with antigen nor with antibody. The solutions which precipitate neither with antigen nor with antibody can obviously be considered to contain neither antibody nor antigen and it can be consequently concluded that the corresponding first precipitate (which has been removed from the solution) contained all the antigens and all the antibody which had been initially present in the system. The mixtures that behave in this way are said to come from the *equivalence zone*, so-called, because in it antigen and antibody are present in such proportions as to precipitate one another completely. This zone of equivalence is near the maximum of the precipitin curve. To the left of it is a region, corresponding to mixtures containing lower concentrations of antigen, called the zone of *antibody excess*. On the right of the equivalence zone is the zone of *antigen excess*.

In the zone of antibody excess, all the antigen is precipitated but not all the antibody, until the equivalence zone is reached. In the region of antigen excess, neither all the antigen nor all the antibody is precipitated since the compounds formed between antigen and antibody are soluble in excess antigen. Consequently supernatants contain free antigen as well as soluble antigen-antibody complex.

In the zone of antibody excess (where all the antigen is precipitated), it is possible to determine accurately the molecular composition of precipitates. It is known that all the antigen is precipitated; the quantity (as mg N) of antigen initially added is known (it is the quantity of antigen-nitrogen that has been added to the solution); the quantity of antibody in the precipitate can be determined by a simple subtraction of the amount of antigen that was added from the measured amount of total precipitate. It is consequently possible to calculate the amount of nitrogen due to antibody in each individual precipitate. If the molecular weight of the antigen and of the antibody is known, the molecular ratio of antigen to antibody in the precipitate can be easily calculated. Such calculations show that the ratio of antigen to antibody varies throughout the range of antigen excess and that antigen will combine with antibody in *multiple proportions.*

The Valency of Antigen and of Antibody

An antigen molecule can combine with more than one molecule of antibody. Because of the analogy to the reaction between atoms, the term valency has been introduced for the combining capacity of antigen and of antibody. This does not imply that the bonds in antigen-antibody complexes are covalent or electrovalent; the linking between antigen and antibody depends most probably on hydrogen bonds.

Just as the valency of antigen is defined as the greatest number of antibodies which can combine with one molecule of antigen so the valency of antibody is defined as the greatest number of antigens capable of combining with one molecule of antibody.

The greatest quantity of antibodies capable of combining with one molecule of antigen is found in extreme antibody excess and gives the *valency* of the antigen. This valency is characteristic for each antigen, depends on the molecular size of the antigen, and is in all known cases, greater than two. The valency of the antibody must be found in the zone of antigen excess, and this information must arise from analysis of the *soluble* antigen-antibody complexes mentioned earlier. This can be achieved in the following manner. An antigen-antibody precipitate is prepared in the range of antibody excess. Its molecular composition is consequently known. To this complex of known composition, enough antigen is added to bring all the antigen-antibody complex into solution. We thus have a solution of known composition. This solution is subjected to some analytical procedure—electrophoresis, or ultra- centrifugation—and a number of components are observed. In electrophoresis, the mobility of the antigen and of the antibody would be known beforehand. If the third substance is seen to move with a mobility other than that of the antigen or of the antibody, it can be quite safely assumed to be a complex of antigen and antibody. The contribution of each component in an electrophoretic analysis can be expressed in terms of the relative proportion of each area in an electrophoretic diagram. Hence the relative proportion of antigen, antibody and antigen-antibody complex in the mixture are known. The composition of the antigen antibody complex can be calculated, since we know exactly how much antibody there is in the mixture.

Such experiments have been carried out by Marrack and Hoch and by Singer and Campbell; the valency of antibody was found to be 2. That is, antibody can combine with no more than 2 molecules of antigen.

From the foregoing experiments, we know that antigen is multivalent and that antibody is divalent. It is thus quite reasonable to assume that the initial

combination of antigen and antibody is followed by a subsequent polymerization of these initial compounds; the valency of both reactants leading to cross-linking between the monomers of antigen and antibody. It has been proposed that it is a lattice, very much like that of an inorganic crystal (3). The only relevant experimental evidence supporting this hypothesis arises out of electron microscope pictures which show a fairly regular pattern of antigen with distances between individual antigen molecules corresponding to the length of an antibody molecule. It can, however, be objected that this observation does not really bear on the problem of a linking between monomers by primary valency forces. Individual monomer molecules, closely packed by precipitation would presumably give a fairly orderly arrangement of the appearance observed in the electron microscope picture. There is, on the other hand, some indirect evidence arising out of the study of combining properties of antigen and antibody which would point to the fact that if polymers are formed the initial monomer, these polymers will be either fairly small and of very irregular shape or would be held together by secondary forces such as Van der Waal forces rather than by the same primary strong bonds which work within the monomers (4).

The Homogeneity of Antigens and the Reaction between Antigen an Antibody in Gels

Let us return to the quantitative precipitin curves and to the precipitates themselves. The determinations can yield an accurate estimate of the quantity of precipitable antibody-nitrogen in a given serum and once this quantity is known, it can of course be used to find the absolute quantity of antigen-nitrogen in an unknown solution. Furthermore precipitin curves can yield valuable information on the properties of both the antigen and the antibody.

So far, it has been assumed that we are dealing with a pure antigen. That is, with a single antigenic component which would not contain any other nitrogenous substance. Unfortunately, the preparation of a homogeneous substance is one of the most difficult tasks of macromolecular biochemistry and usually one is dealing with an antigen which consists of more than a single component. Such an antigen, when used to immunize a rabbit, would obviously yield more than one antibody corresponding to each single antigen. The interaction of the mixture of antigens with their corresponding antibodies would thus yield a number of independent precipitating systems and thus a number of curves for each antigen-antibody system contained in the mixture. Since one is measuring nitrogen and since each system will contribute nitrogen, an additive curve consisting of the individual quantitative precipitin curves will be obtained. It is clear that if this is the case, it is very unlikely that all the different individual equivalence zones corresponding to each individual antigen-antibody system will be in the same position. Thus, some supernatants will be found in which precipitation will occur whether antigen or antibody is added. This can be used as a criterion for the purity of an antigen. It is, however, impossible with this technique to arrive at any statement about the number of antigenic impurities or about the relative proportion of the varying impurities.

We have so far discussed the action of antigen and antibody in the bulk of a solution. The reaction between antigen and antibody can, however, also be observed in a transparent agar gel (5,6). The antibody is incorporated into the gel, the gel is poured into a tube and the antigen is layered above the gel. Thus at the beginning there is no antigen in

the gel and as time passes the antigen diffuses through the gel.

It can be assumed that the concentration of antibody within the gel remains constant. On the other hand, the concentration of the antigen throughout the tube must vary with time, with the distance (in the tube) from the interface, and it must depend on the diffusion constants of the antigens.

After some time an opaque disk, consisting of a precipitate of antigen and antibody, can be observed within the agar; this zone moves towards the bottom of the agar column.

One antigen and its corresponding antibody can give rise to only one zone. The interpretation of all agar diffusion experiments depends on the validity of this assumption and it is therefore important to examine it closely. It is intuitively obvious, that the concentration of the antigen varies in a continuous manner from the top of the agar column downwards. The antibody concentration, on the other hand, remains the same throughout the length of the column since we have incorporated antibody into the gel and have stirred the gel thoroughly so that every unit volume of the gel contains the same amount of antibody. Consequently, the conditions that prevail throughout the length of the gel are very similar to the conditions prevailing in an assembly of test tubes set up to measure quantitative precipitin test in bulk and containing mixtures identical in antibody concentration and varying in antigen concentration. We can thus assume that if a precipitation zone is laid down in an agar column, the conditions inside the column will be similar to those described in a quantitative precipitin test: progressing from low to high antigen concentrations the total amount of antigen-antibody precipitate is at first increased and subsequently, beyond the maximum, it decreases again. On this basis it can be assumed that the density of a zone laid down by a single antigen reaction with a single antibody would have one single maximum of density. Consequently one antigen and one antibody react in agar with the deposition of only one zone within which the optical density (i.e., the quantity of material precipitated) increases, reaches a maximum and then decreases. The only difference between the precipitin systems set up in a series of test tubes and the precipitin system set up in an agar column consists in the fact that while we can vary the concentration of antigen from test tube to test tube in any *arbitrary* manner, the variation of antigen concentration along the length of an agar tube is determined by the laws of diffusion. While there is thus no essential difference between reaction in bulk and in gel, when a single antigen-antibody system is involved, there is quite an important difference when several antigens and, therefore, several antibodies interact in the same solution.

When discussing the quantitative precipitin test in the bulk of a solution, we came to the conclusion that several independent antigen-antibody curves would be observed as a single summed curve. In agar the individual antigen-antibody systems can be observed separately. The distribution of each antigen will be governed by the laws of diffusion and will, therefore, depend on the concentration and on the diffusion constant of each antigen. Both these values will be different for each antigen. Therefore the precipitating zone in the agar column for each of several antigens will occur at a different height and a number of zones will be observed in different positions of the agar column, each corresponding to a single antigen-antibody system. Precipitation and diffusions in agar thus permit the enumeration of the antigens present in a complex mixture. Sensitive as this method is, it may not show all of the antigens present. This

will depend mainly on the concentration of each of the antibodies corresponding to the antigens, and, of course, also on the concentration of the antigens themselves. Clearly, if the concentration of the antigens or of the antibodies is too low to give the precipitate that can be seen, the zones corresponding to the antigen will not be recorded.

The interaction of antigen and antibody in agar was first understood by Oudin in 1946 and analyzed by him over the next twelve years. It has become a valuable tool, allowing the analysis of a complex mixture for the minimum number of antigens contained in it. Shortly after Oudin's first revolutionary paper on the subject, Ouchterlony tried a somewhat different experimental arrangement for the observation of the interaction between antigen and antibody in gels. He prepared a flat slab of agar in a Petri dish, made two wells in the agar, filled one with antigen and the other with antibody and observed the antigen-antibody precipitates formed when antigen and antibody, after diffusing, reacted with one another.

Oudin, who refers to his own system as one of "simple diffusion", has referred to that of Ouchterlony and to other related modifications as the "method of double diffusion". Both methods can be adapted so as to compare several antigens or several different mixtures of antigens in their reaction with the same antiserum or alternatively, to compare the reactions of different antibodies with the same antigen. The influence and the importance of these techniques in a variety of fields of research can hardly be exaggerated and it can be claimed that agar diffusion has been to immunochemistry and to other fields of quantitative biology, of the same importance as paper chromatography has been to analytical chemistry and biochemistry.

We have thus seen that antigen and antibody combine in solution, that the complex becomes insoluble by some process of aggregation, that antigen can combine with several molecules of antibody and that antibody can combine with two molecules of antigen. The precipitation of antigen and antibody and the reaction in gels can be used for the examination of complex mixtures of antigens. This can be used in many biological studies wherever cells or fluids as the result of some change, lose or gain antigens.

Specificity, Autoantibodies and the Inheritance of Immunological Responsiveness

The dynamic aspect of immunochemistry, the synthesis of antibody, should be the concluding chapter. Unfortunately too little is known about this to justify such a choice. Antibodies are almost certainly formed in plasma cells but their mode of formation is still in the realm of conjecture. On the other hand some unifying observations may help to co-ordinate some aspects of the immune response and we shall try to examine them.

Antibody formed in response to an antigen, reacts with this antigen and virtually with no other. This phenomenon is referred to as specificity (7). A weak reaction can, however, sometimes be obtained with a substance other than the antigen which has given rise to the antibody. This "cross reaction" has been studied in model experiments as will now be described.

A small organic molecule is conjugated to an antigen, frequently through an azo linkage. The resulting antibody contains molecules which are specifically adapted to this small organic molecule. This can be shown by conjugating the same organic molecule to another antigen and demonstrating that this conjugated *second* antigen interacts with antibodies

to the *first* conjugated antigen. Furthermore even though an antibody to para-azo benzene sulphonic acid reacts most strongly with p-azo benzene sulphonic acid, a weaker reaction is obtained when an antigen is used that contains meta-azo benzene sulphonic acid and a still weaker reaction when the antigen contains ortho-azo benzene sulphonic acid. Thus the specificity of the antibody is great enough to distinguish between the positions of the two substituted groups on the benzene ring. It is even capable of distinguishing between halogenated benzene rings in which one benzene ring might contain fluorine, another bromine, another iodine. Experiments of this kind have shown antibodies to be capable of distinguishing differences of as little as 1 Angstrom in molecular size (7).

With natural polysaccharides detailed studies have revealed that the antibody is adapted to a relatively small surface area of the antigens and that the largest sequence of sugars to which an antibody has been found to be adapted is no more than five sugars but that usually the determinant area with which the antibody combines and against which it is formed, is one sugar linkage. It is probable that this is also true of proteins, that no more than five and as little as two amino acids may act as determinant sites on the protein. Most proteins contain all the amino acids of which there are 20. This means that the chance of finding any two particular amino acids side by side on an amino acid chain containing very large numbers of these molecules must be very high, and consequently nearly all proteins should contain overlapping sequences of amino acids and should cross-react. That this is not observed represents our first clue. One would suspect the existence of a process which limits the cross-reactivity between protein antigens. This may be linked with another limitation, the absence of the formation of antibody to the antigens of the body itself, and thus to a large number of different proteins synthesized by the organism. Before we can pursue this particular clue we have to consider a number of exceptions to this mechanism of inhibition. The older literature contains sporadic references to the production of autoantibodies in response to the injection of certain homologous tissues, previously exposed *in vitro* to bacterial toxins or to the direct action of bacteria. On the whole, this early work was hard to repeat and the induction of antibodies to autologous tissue was only successful in a very small number of animals injected. Recently this field has entered into a new phase. Here again a new technique became the pace-maker of a new development. Freund (8) described an adjuvant consisting of Bayol F, Aracel A and heat killed mycobacteria which potentiated the antigenic power of most antigen macromolecules. It was soon found that when certain homologous tissues incorporated in Freund's adjuvant were injected subcutaneously, changes in the corresponding tissues could be observed in the body. Antibodies were in this way induced to testicular tissue, adrenal tissue, to thyroglobulin and to nervous tissue, and such antibodies caused respectively azoospermia, degeneration of the adrenal gland, thyroid deficiency disease and various forms of neuritis and encephalitis.

Recent discoveries have made it quite clear that these autoantibodies are not just a curiosity due to an extreme experimental provocation. It was shown that in Hashimoto's disease a very potent circulating antibody to thyroglobulin plays a part in the development of the disease.

It seems thus evident that a certain number of tissues can give rise to autoantibodies and that all these tissues contain antigens which are localized, do not

circulate, and are hence not widely distributed through the body. This is particularly clearly illustrated in Hashimoto's disease, since in this condition the thyroglobulin which otherwise is retained within the lumen of the follicles of the thyroid gland appears to have leaked into the circulation, giving rise to antibody formation and to have thus triggered off the disease process. We are consequently led to think that the free circulation through the body of an antigen may at some stage prevent the formation of antibody to this tissue. These facts provide our second clue.

If a group of animals is injected with the same antigen and the antibody response to this antigen is measured after a standard time, it is found that the quantity of antibody produced by different animals varies widely. If two animals which both produce a high titre are mated, it is found that their offspring produce a high titre and that they differ significantly from the randomly bred population. Conversely, if animals are bred which have a low titre then offsprings are obtained which respond very poorly (14).

In recent experiments we have encountered an even more fascinating aspect of this (9). We found that bovine ribonuclease, which is a reasonably good antigen in wild type rabbits gave rise to practically no measurable antibody in another strain of rabbits, white Himalayans. However, other antigens, for instance human albumin or sulphonic azo human albumin, provoked in white Himalayan rabbits as high an antibody titre as in wild type rabbits. It has thus to be concluded that not only is the antibody response to a given antigen inherited, but the antibody response due to different antigens is independently inherited. It is clear, from what has been said earlier on, that an enormous variety of substances can be antigens and it is almost inconceivable that the response to each of these antigens should be independently and directly inherited. There just are not enough genes. In this difficulty we find our third clue.

Somehow these three clues have to fit together and how they may fit together became apparent as a consequence of the discovery of acquired immunological tolerance. This discovery predicted by Burnet (10) in 1949 forecast by work by Owen (11) in 1945 was finally made by Medawar in 1953 (12) when he found that hetero-tissue grafts which would have been rejected within a few days could become acceptable when the animal grafted had been injected by tissue from the donor before birth. It was soon found that when a purified protein was injected just before or just after birth, the animal so injected could not make an antibody to this particular antigen in later life.

Human albumin, for instance, was injected into rabbits during the first ten days after birth; 3 or 4 months later the animals were again injected with albumin. The human albumin circulated for a very long time and disappeared, finally from the circulation after the 2nd slow and steady phase of elimination had persisted for 2 or 3 weeks (see p. 138). The rabbits had thus lost the ability to make antibody to human albumin, moreover, when an animal thus rendered tolerant to human albumin was later on immunized with a closely related but slightly modified molecule, namely azo human albumin, it responded not at all or with a very low titre to this azo human albumin. The few animals which responded to the conjugated azo human albumin produced an antibody which was almost exclusively adapted to the azo group (13).

Azo albumin is normally a good antigen; under the experimental conditions described, it became a bad antigen.

We can now consider our three clues in the light of this observation. We can, first of all, discern a limiting mechanism which would result in the specificity of the antibody response to a protein. Each protein would carry a very large number of sites which are potentially capable of giving rise to antibody. Any sequence of two or three amino acids would constitute such a site, but when such a sequence of amino acids has already been circulating in embryonic life or immediately after birth, then this particular site would be excluded as an inducer of antibody synthesis, and, of course, in any developing animal a large number of amino acid sequences are present within autologous proteins of the animal. Therefore, the antibody-response would be limited to foreign proteins containing amino acid sequences "new" to the antibody forming cells and absent from any of the circulating proteins of the animal. Hence only a few of all the amino acid sequences would be able to elicit an antibody response. The high specificity of the antibody response to foreign protein and the rarity of cross-reaction between two foreign proteins could then be understood. Not all amino acid sequences but only a very few could act as antigenic determinants, and the likelihood that two unrelated proteins would share such an amino acid sequence would be correspondingly small. Autoantibody formation would be possible to such antigens as were absent from the circulation before or just after birth.

The inheritance of the antibody-response would be similarly explained. The intensity of the antibody response would depend on the number of sites on a molecule which can, in fact, give rise to antibody. The number of sites on a given molecule which could elicit an antibody response would depend on the *amino acid sequences on the autologous proteins.* These would be genetically determined. Thus the number and amino acid sequences on a foreign protein which could act as antigenic determinants would not be quite the same in two genetically different rabbits. In two animals the antibody response to, say human albumin, could differ by a factor of six if albumin were to share all but *one* group of its potential antigenic sites with autologous proteins in one animal, and in the other animal would share all but *six* sites with autologous proteins. Thus we can understand the inheritance of the antibody response in terms of the genetic control of the synthesis of autologous proteins.

We have thus seen that unifying factors are arising in a bewildering number of phenomena. There emerges as a consequence of this unification, another problem on the next level of analysis and in which, so far, little fundamental information is available. This is the basic problem of the control and synthesis of protein, of the mechanism and nature of the synthesis of the antibody and of the processes guiding molecular synthesis so as to allow the resulting antibody to combine with the antigen which has elicited its synthesis. This, no doubt, will be the focus of the challenging tasks which will be attempted by many research workers in the coming years.

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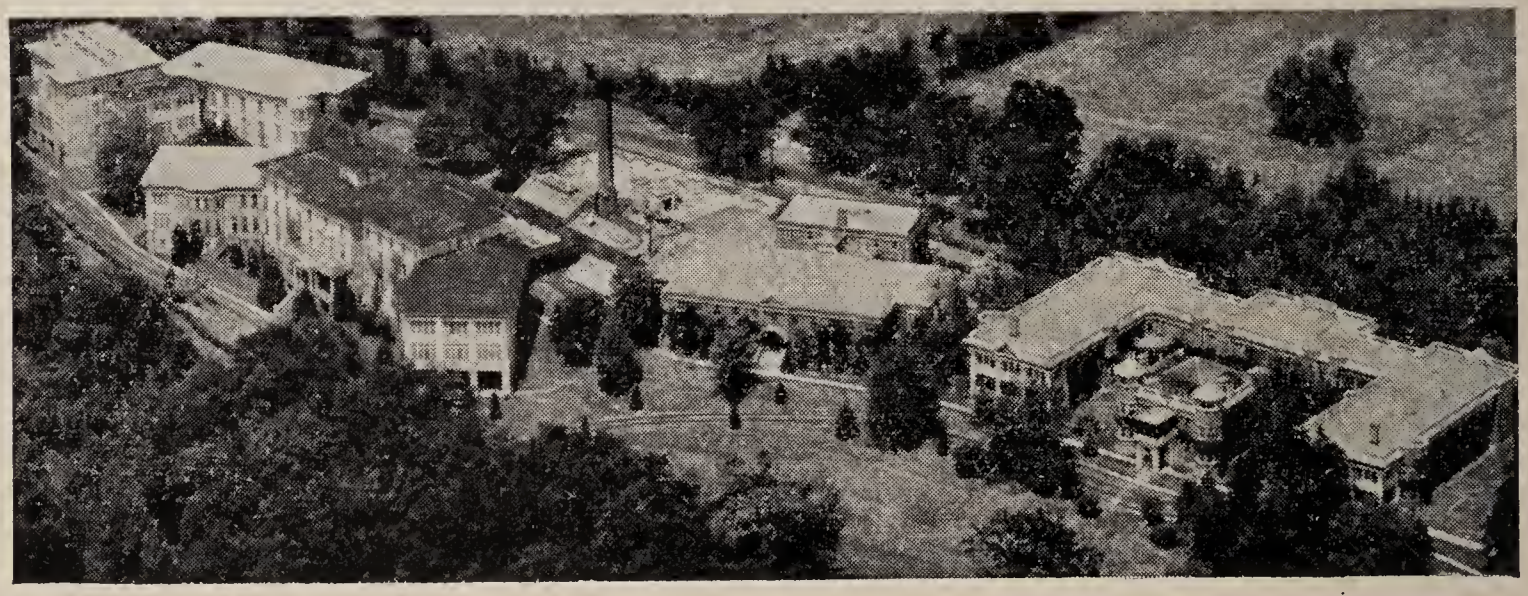

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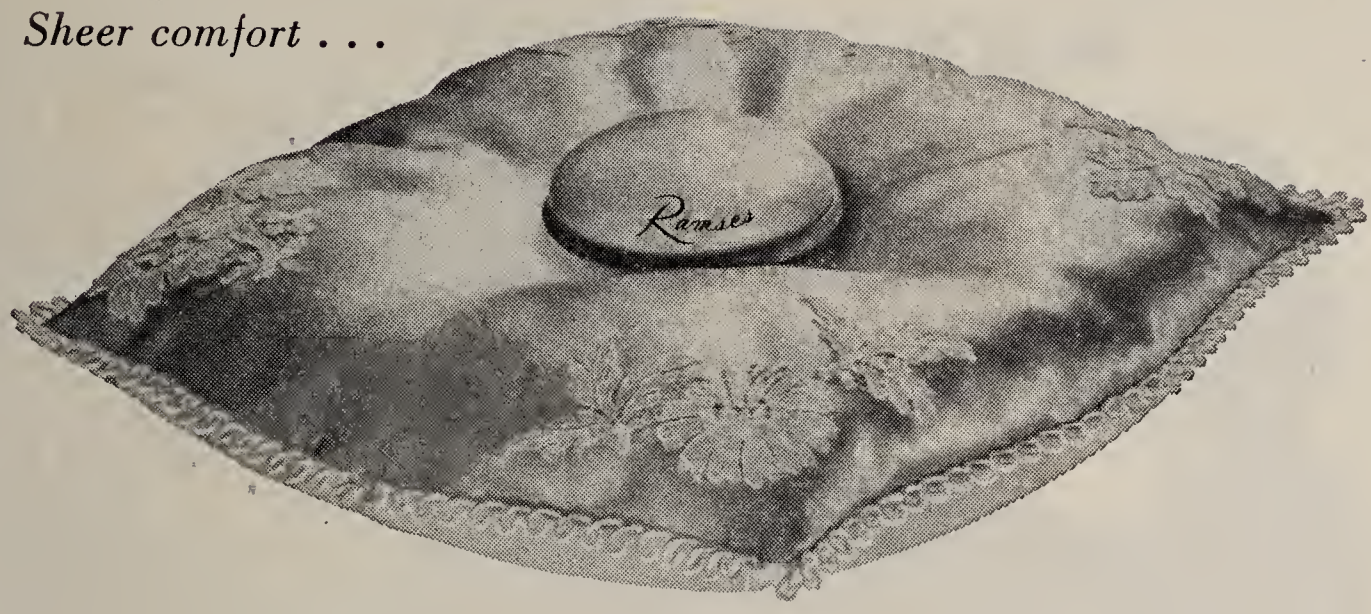

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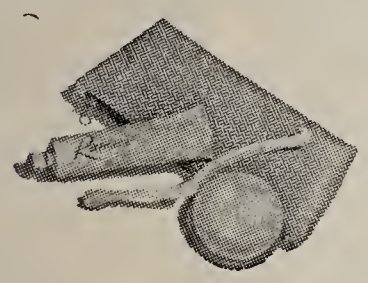

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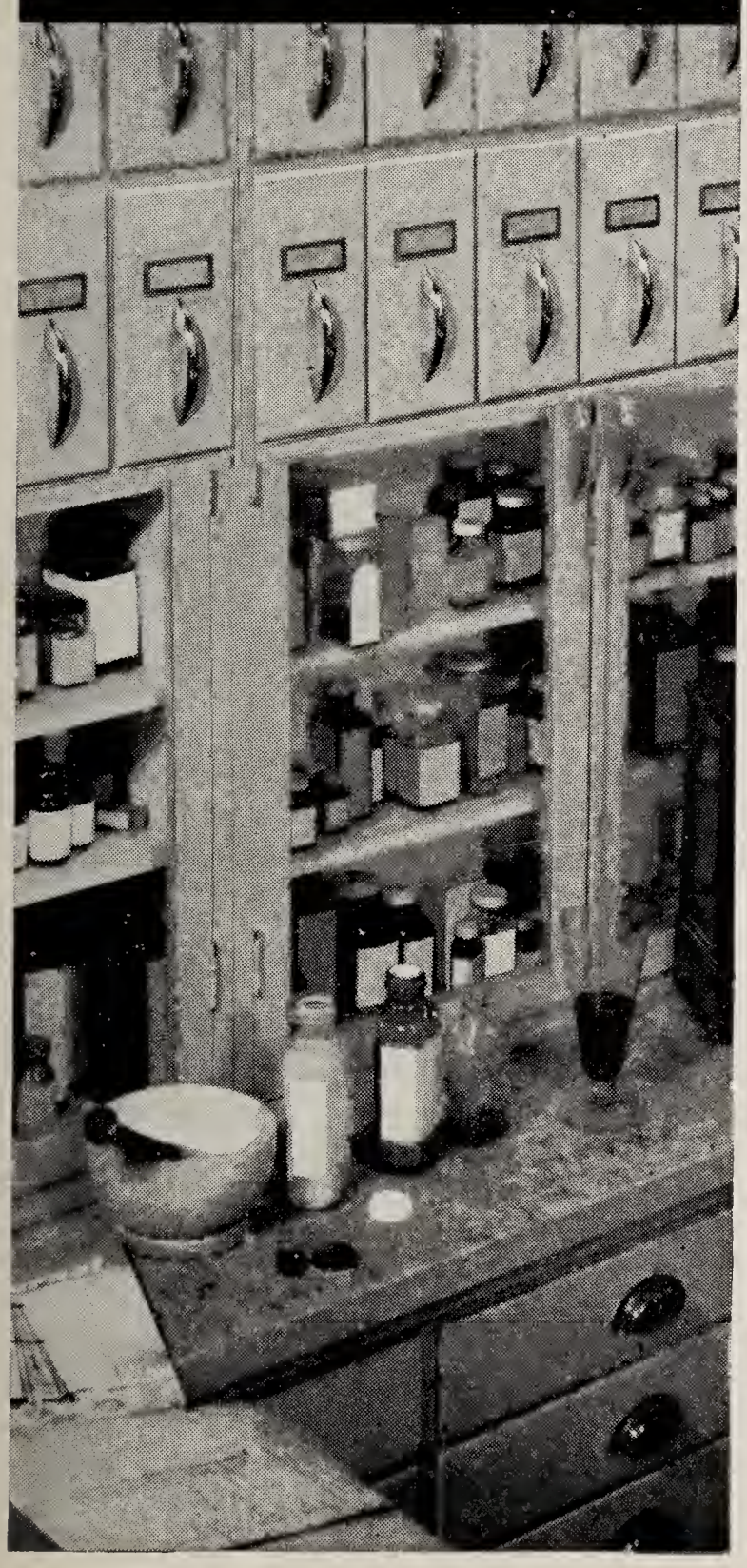

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